# Tracking and identifying wastewater toxicants and assessing their biodegradation products

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Wastewater effluent from wastewater treatment plant (C ALAIN LE BOT/GAMMA/SKOY)

# Contents

# List of original publications

This thesis is based on the following publications, which are referred in the text by Roman numerals (I-V).

I Pessala P, Schultz E, Nakari T, Joutti A & Herve S (2004) Evaluation of wastewater effluents by small-scale biotests and a fractionation procedure. Ecotoxicology and Environmental Safety, Vol. 59, 263-272.

II Pessala P, Schultz E, Luukkainen S, Herve S, Knuutinen J & Paasivirta J (2004) Lignin as the cause of acute toxicity in pulp and paper mill effluents? Pulp and Paper Mill Effluent Environmental Fate & Effects, (eds.) Borton, D.L, Hall T.J, Fisher R.P. & Thomas J.F. DEStech Publications, Inc. Lancaster, USA p 319-330.

III Pessala P, Schultz E, Kukkola J, Nakari T, Knuutinen J, Herve S & Paasivirta J (2008) Biological effects of lignin derivatives and HMW compounds of laboratory-scale ECF and TCF effluents. *Submitted to Ecotoxicology and Environmental Safety.*

IV Paasivirta J, Knuutinen J, Kukkola J, Pessala P, Schultz E and Herve S (2005) Multivariate statistics of the pyrolysis products of high molecular components in pulping wastewaters to explain their toxicity. Environmental Science and Pollution Research, Vol. 12, 375-380.

V Pessala P, Keränen J, Schultz E, Nakari T, Karhu M, Ahkola H, Knuutinen J, Herve S, Paasivirta J & Ahtiainen J (2008) Evaluation of biodegradation of nonylphenol ethoxylate and lignin by combining toxicity assessment and chemical characterization. *Submitted to Chemosphere*.



**Author's contribution:**

I The author was responsible for the overall planning of the study, data analysis and writing of the paper. The author was responsible for performing the microbiological tests and the overall effluent fractionations. Corresponding author.

II The author was responsible for planning the study together with the co-authors. She was responsible for the overall data analysis and writing of the paper. The author was responsible for performing the luminescent bacteria tests. Corresponding author.

III The author was responsible for planning the study together with the co-authors. She was also responsible for the overall data analysis and writing of the paper. The author carried out the luminescent bacteria tests. Corresponding author.

IV The author was responsible for initiating the study to characterize the toxicity causing chemical structures. She participated in the writing of the paper.

V The author was responsible for planning the study design with Jukka Ahtiainen and Eija Schultz. She was responsible for the overall data analysis and the writing of the paper and for performing hER yeast tests and ATP tests.

# Abbreviations

| | |
|---|---|
| AOX | Absorbable organic halogens |
| APE | Alkylphenol ethoxylates |
| ASTM | American Society for Testing and Materials |
| ATP | Adenosine triphosphate |
| BOD | Biological oxygen demand |
| cDNA | Complimentary DNA |
| COD | Chemical oxygen demand |
| DNA | Deoxyribonucleic acid |
| DOC | Dissolved organic carbon |
| DR-CALUX | Dioxin responsive chemical-activated luciferase gene expression |
| ECF | Elementally chlorine free |
| EC | Effective concentration |
| EDA | Effect-directed analysis |
| ELISA | Enzyme-linked immunosorbent assay |
| EPA | Environmental Protection Agency |
| ER | Estrogen receptor |
| ER-CALUX | Estrogen receptor chemical-activated luciferase gene expression |
| ERA | Environmental risk assessment |
| EROD | Ethoxyresorufin-O-deethylase |
| ETr | Electron transport |
| EU | European Union |
| EVB-DVB | Ethylvinylbenzene-divinylbenzene |
| $FMNH_2$ | Reduced flavin mononucleotide |
| FMN | Flavin mononucleotide |
| GC-FID | Gas chromatography – flame ionisation detector |
| GC-MS | Gas chromatography – mass spectrometry |
| GFP | Green fluorescent protein |
| GM | Genetically modified |
| H-NMR | Proton nuclear magnetic resonance |
| HMW | High molecular weight |
| HPLC | High performance liquid chromatography |
| HRT | Hydraulic retention time |
| ISO | International Organization for Standardization |
| LMW | Low molecular weight |
| LOEC | Lowest observed effect concentration |
| MTBE | Methyl-*tert*-butyl ether |
| MWCO | Molecular weight cut-off |
| NAD(P) | Nicotinamide adenine dinucleotide (phosphate) |
| NAD(P)H | Reduced nicotinamide adenine dinucleotide (phosphate) |
| NOEC | No observed effect concentration |
| NPDES | National Pollutant Discharge Elimination System |
| NPE | Nonylphenol ethoxylate |
| OECD | Organisation for Economic Co-operation and Development |
| PCA | Principal component analysis |
| PEC | Predicted environmental concentration |
| PNEC | Predicted no-effect concentration |
| PS-DVB | Poly(styrene-divinylbenzene) |

| | |
|---|---|
| REACH | Registration, evaluation, authorisation and restriction of chemicals |
| RET | Reverse electron transport |
| RNA | Ribonucleic acid |
| SMP | Sub-mitochondrial particle |
| SMLR | Stepwise multiple linear regression |
| SPE | Solid phase extraction |
| TCF | Totally chlorine free |
| TIE | Toxicity identification evaluation |
| TRE | Toxicity reduction evaluation |
| U.S. EPA | U.S. Environmental Protection Agency |
| UV | Ultraviolet |
| WET | Whole effluent toxicity |
| WFD | Water Framework Directive |
| WWTP | Wastewater treatment plant |
| YES | Yeast estrogen screen |

# Abstract

Huge amounts of wastewaters of varying composition are treated and discharged annually from wastewater treatment plants. In Finland approximately 500 million cubic meters per year are discharged from municipal wastewater treatment plants alone. Wastewater treatment plants use chemical, physical and biological unit operations and processes in removing solids, nutrients, and harmful compounds from the wastewater prior to discharging. However, treated wastewaters discharged into the receiving waterway still include a wide variety of potentially harmful substances despite the extensive treatment processes.

Effluent discharges are controlled by monitoring and setting consent conditions based on chemical and physical parameters. In addition, legislation and/or uniform guidelines on discharge limits based on biological parameters have been implemented in some countries (e.g. in the USA and Germany). Effect directed analysis, meaning the combination of physico-chemical fractionation methods and biological tests to reveal the biologically active components, has been developed especially in the USA to track harmful components from discharges. In Finland, however, biological methods have not been used routinely in establishing effluent discharge limits.

Ecotoxicological tests are biological methods used to assess the effects on, for example, growth, reproduction and behaviour of organisms. Traditionally ecotoxicological effect studies have relied on whole organisms, e.g. fish, and have therefore required quite long test durations and large test volumes. Today, however, the trend has been towards economically and ethically more viable test methods. Thus, increasing interest has been paid on the development, validation and use of small-scale test methods, which are rapid, need small test volumes and can be automated at least partially. In this study the focus was on applying several small-scale methods for assessing wastewater effluents and to elaborate effect directed assessment methods. This was done to provide tools for evaluating wastewater effluents or other complex aquatic samples with biological methods, and for characterising and identifying their toxicity causing compounds and structures.

Screening of municipal and industrial wastewater effluents confirmed that Finnish wastewater treatment plants are efficient in removing components that might cause acute effects in whole organisms. However, indications of estrogenic and genotoxic potential were detected. It also showed that accidental releases are potent in discharging biologically harmful compounds. In the later steps of this study, toxicity causing components were tracked from effluents originating from a pulp and paper mill. Lignin was identified as the main toxicant, when using an *in vitro* screening method based on reverse electron transport (RET) of mitochondrial membranes. Chemical structures that could explain the toxicity of lignin were characterized by combining chemical, biological and statistical methods. Also impacts of biodegradation on the toxicity of organic compounds appearing in wastewater effluents (lignin and nonylphenol ethoxylates) was studied. It was shown that combining biological effect assessment directly with biodegradation tests is possible when sensitive small-scale bioassays are used. Effect directed assessment methods as used for effluents could be used also in biodegradation studies. Finally, a tiered approach combining chemical, biological and statistical methods is proposed for evaluating biologically active organic compounds appearing in wastewater effluents.

# Tiivistelmä (Abstract in Finnish)

Suuret määrät koostumukseltaan vaihtelevia jätevesiä käsitellään ja lasketaan jätevedenpuhdistamoilta vuosittain. Suomessa päästöt ovat noin 500 miljoonaa kuutiometriä vuodessa pelkästään kunnallisilta jätevedenpuhdistamoilta. Jätevedenpuhdistamot käyttävät kemiallisia, fysikaalisia ja biologisia yksikköoperaatioita ja -prosesseja poistaessaan kiintoainetta, ravinteita ja haitallisia yhdisteitä jätevesistä. Kattavasta jätevedenkäsittelystä huolimatta vastaanottavaan vesistöön päästettävät jätevedet sisältävät monia erilaisia potentiaalisesti haitallisia aineita.

Jätevesipäästöjä kontrolloidaan tarkkailemalla ja asettamalla lupaehtoja perustuen kemiallisiin ja fysikaalisiin parametreihin. Lisäksi eräissä maissa (esim. Yhdysvalloissa ja Saksassa) on pantu täytäntöön lainsäädäntöä ja/tai yhtenäisiä ohjeistuksia biologisille parametreille perustuville lupaehdoille. Erityisesti Yhdysvalloissa on kehitetty vaikutukseen perustuvaa analyysiä haitallisten aineiden jäljittämiseen jätevesistä. Tällä tarkoitetaan fysikaalis-kemiallisten fraktiointimenetelmien yhdistämistä biologisiin testeihin biologisesti aktiivisten komponenttien paljastamiseksi. Suomessa biologisia menetelmiä ei ole käytetty rutiininomaisesti lupaehtoja laadittaessa.

Biologisilla menetelmillä tarkoitetaan tässä yhteydessä ekotoksikologisia testejä, joita käytetään arvioimaan vaikutuksia esimerkiksi organismien kasvuun, lisääntymiseen tai käyttäytymiseen. Perinteisesti ekotoksikologiset vaikutustutkimukset ovat perustuneet kokoeliöihin, esim. kaloihin, ja ovat siksi vaatineet melko pitkän testiajan sekä suuret testitilavuudet. Nykyään suuntaus on kuitenkin ollut kohti taloudellisesti ja eettisesti toteuttamiskelpoisempia testimenetelmiä. Siksi lisääntyvää mielenkiintoa on kohdistettu pienen mittakaavan testien kehittämiseen, validointiin ja käyttöön. Tällaiset testit ovat nopeita, vaativat pienen testitilavuuden ja ovat ainakin osittain automatisoitavissa. Tässä tutkimuksessa tarkoituksena oli soveltaa useita pienen mittakaavan testejä jätevesien arviointiin ja edelleen kehittää menetelmiä vaikutukseen perustuvaa analyysiä varten. Tarkoituksena oli tuottaa työkaluja jätevesien tai muiden monikomponenttisten vesinäytteiden arviointiin biologisilla menetelmillä sekä haitallisten aineiden ja rakenteiden karakterisointiin ja identifiointiin.

Kunnallisten ja teollisuuslaitosten jätevesien tutkiminen vahvisti sen, että suomalaiset jätevedenpuhdistamot ovat tehokkaita poistamaan komponentteja, jotka saattaisivat aiheuttaa akuutteja vaikutuksia kokonaisissa eliöissä. Kuitenkin havaittiin viitteitä estrogeenisestä ja genotoksisesta potentiaalista. Lisäksi häiriöpäästöt osoittautuivat potentiaalisiksi biologisesti haitallisten aineiden lähteiksi. Tutkimuksen myöhemmässä vaiheessa jäljitettiin myrkyllisyyttä aiheuttavia komponentteja sellu- ja paperitehtaan jätevedenpuhdistamon jätevedestä. Ligniini tunnistettiin pääasialliseksi myrkyllisyyden aiheuttajaksi käytettäessä mitokondriaalisilla membraaneilla tapahtuvaan elektroninsiirtoon perustuvaa *in vitro* -testiä (RET-testi). Kemiallisia rakenteita, jotka voisivat selittää ligniinin myrkyllisyyttä, tutkittiin yhdistämällä kemiallisia, biologisia ja tilastollisia menetelmiä. Lisäksi tutkittiin biohajotuksen vaikutusta jätevesissä esiintyvien orgaanisten yhdisteiden (ligniini ja nonyylifenolietoksylaatit) myrkyllisyyteen. Tutkimuksessa osoitettiin, että on mahdollista yhdistää biologisia vaikutusarviointeja suoraan biohajoavuustesteihin käyttämällä herkkiä pienenmittakaavan biotestejä. Samanlaista vaikutuksiin perustuvaa analyysiä, jota käytettiin jätevesille, voitaisiin käyttää myös biohajoavuustutkimuksissa. Lopuksi jätevesissä esiintyvien biologisesti aktiivisten orgaanisten yhdisteiden arviointiin ehdotetaan monitasoista, kemiallisia, biologisia ja tilastollisia menetelmiä yhdistävää lähestymistapaa.

# 1 Introduction

## 1.1 Ecotoxicological assessment

### 1.1.1 Concept of ecotoxicology

Ecotoxicology in its broader definition is a science that deals with the fate and the effects of substances in the environment. The fate of substances, be they natural or xenobiotic, means the behaviour (e.g. dissociation, biodegradation, accumulation) of the substances in the environment. Ecotoxicological effects mean the interference of substances with living organisms. Exposure to harmful substances can cause acute or chronic effects in organisms. The effect may be directed at, for example, the organism's genome or vital biochemical functions. The possible environmental fates and effects of chemicals and environmental samples are often studied in laboratory-scale, yet studies using mesocosms or macrocosms, as well as studies directly in the nature, are also possible.

Ecotoxicological assessment provides tools and data for environmental risk assessment (ERA), whereas toxicological data is used in assessing health risks. ERA is an integral part of environmental impact assessment and it can be used to predict possible ecological risks of, for example, wastewaters. Ecotoxicological data is used to calculate predicted environmental concentrations (PEC) and predicted no-effect concentrations (PNEC), which are key parameters of ERA (EC, 2003). On the other hand, ERA can be used to evaluate sites where contamination has already happened. In such cases laboratory tests using the contaminated matrix or biomarker studies from exposed organisms can be used for assessing the significance of pollution and the required remedial processes. Risk assessment of chemicals leans also on ecotoxicological data. For example the new European chemicals legislation, the REACH regulation (Registration, evaluation, authorisation and restriction of chemicals), requires chemical safety assessments based on ecotoxicological data for all substances produced or imported in quantities of 10 or more tons per year (EC, 2006). Chemicals that do not pass certain criteria are subject to restrictions or their sale and use can even be prohibited.

Although risk assessments of pure chemicals and environmental samples do differ, the ecotoxicological test set-ups may be rather similar. However, when concerning methods for environmental samples, especially the possible effects of the matrix and sampling conditions have to be carefully considered. Chemical risk assessments are usually carried out on single substances, although in the environment they are functioning as parts of compound mixtures. International harmonisation and standardisation of ecotoxicological methods is carried out by the Organisation for Economic Co-operation and Development (OECD) and the International Organization for Standardization (ISO), OECD concentrating on chemical testing and ISO on environmental analysis. A lot of research work is also being carried out using non-standardised methods – some of which may some day become standards.

Effects of chemicals and other samples are often delineated by a dose-response relationship meaning that the response is related to the chemical dose, i.e. to exposure to the chemical. Varied methods for obtaining and calculating dose-response relationships are used, but the principle and ultimate task is the same: quantification of the harmful effect and relating it with the exposure. Thus, ecotoxicological effect studies are usually carried out by exposing a biological receptor for a series of sample dilutions. Effects of these diluted samples are compared to an unexposed set-up. As a result, usually EC-values and/or LOEC and NOEC values are

calculated depending on the test set up and purpose. EC50 value (effective concentration value) represents the concentration of the studied sample that causes an effect on half (50 %) of the tested system. Also other EC-values (e.g. EC10 and EC20) can be calculated. Calculation of NOEC-values (no observed effect concentration) aims at finding out the test concentration causing statistically no significant difference in the exposed and unexposed test systems. The lowest observed effective concentration (LOEC) is the test concentration one step higher from the NOEC-value. Hence, the selected test concentrations with the factor used between the successive concentrations are of major importance. In ecotoxicological testing positive controls (reference compounds) are usually also included in the test design, in order to ascertain the validity of the particular test occasion.

Different endpoints can be used for the quantification of harmful effect. Reproduction, growth and survival are endpoints that have been traditionally used in whole organism tests. Many of these types of responses can be detected by eye and the test durations are quite long, especially if they are to cover the organism's full life cycle (from several days to months or even years). On the other hand, methods that are based on changes in certain cellular or sub-cellular reactions and activities, as well as on microbes as test organisms, have started to be used more and more. Typical for these assays is their small-size, short test duration and the need for special instrumentation to detect the response. Development of such new test methods has been driven by the wish to diminish the use of vertebrate tests. One major driver today is REACH, which necessitates the reduction of vertebrate testing (EC, 2006). A wide variety of rapid small-scale tests have been developed as possible alternatives for the traditional tests.

In addition to the possibility to reduce the use of vertebrates, small-scale tests are also quite fast and (semi-)automation of the procedures is often possible. Thus, besides the ethical considerations, small-scale methods are also economically more feasible and thus more potential for routine control and assessment use. They are often relatively sensitive and thus also reduce the risk of the biological effects being underestimated (Blaise, 1998). Results from small-scale tests are often compared to traditional whole organism tests. It is not yet clear how well small-scale tests resemble the responses in whole organisms in actual environmental conditions.

Images of some test organisms or systems commonly used for studying biological effects are presented in Figure 1. Different types of ecotoxicological methods for assessing biological effects, both standardized and non-standardized, are described in more detail in the following sub-chapters.

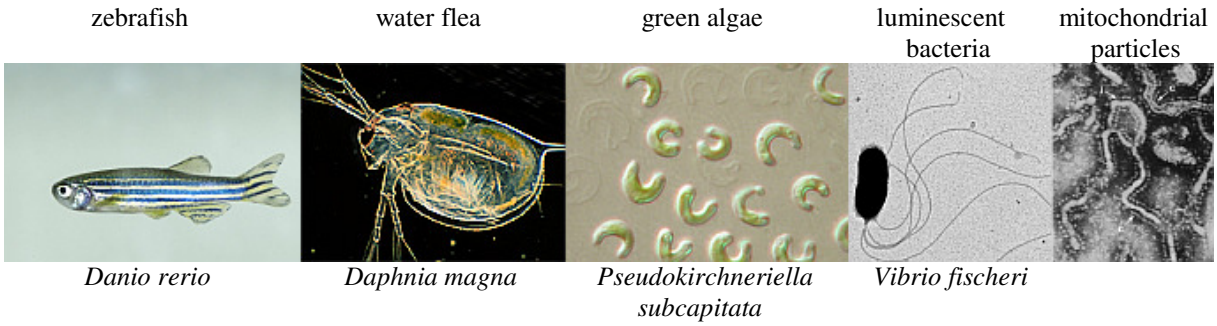



**Figure 1.** Common test organisms or systems used in ecotoxicological effect studies (not to scale). (Pictures: zebrafish ©Dries Knapen, http://www.focusonnature.be; green algae ©Crown, www.cefas.co.uk/media/46903/p-sub-pic-1.jpg; water flea, ©Jukka Järvi, Finnish Environment Institute; luminescent bacteria ©Deborah Millikan, http://www.pnas.org/cgi/content-nw/full/102/8/2673#FIG2; Mitochondrial particles from Parsons, D. F., 1963. Science 140:985. Reprinted with the permission from AAAS)

### 1.1.2 Traditional tests with whole organisms

Use of test organisms from different trophic levels has been considered to be most suitable for environmental protection. Thus, the traditional whole organism methods comprise a suit of organisms from different trophic levels and environments. Whole organisms used in ecotoxicological effect testing range from unicellular organisms (e.g. bacteria) to vertebrates (e.g. fishes) and many such tests methods have been harmonised already for decades. In addition, many of the unicellular whole-organism methods have been miniaturized and are performed as small-scale applications today. There are different assays based on terrestrial, aquatic and sediment organisms, as well as for freshwater and marine environments. In this context the focus is mainly on aquatic freshwater organisms.

Algae are widely used as representatives of the trophic level of primary producers in the ecotoxicity tests. Test protocols have been standardized and such methods exist for both freshwater and marine algae. The freshwater test is based on the growth inhibition of unicellular planktonic green algae *Pseudokirchneriella subcapitata* (former *Raphidocelis subcapitata* and *Selenastrum capricornutum*) and *Scenedesmus subspicatus* (ISO 8692, 1989). The marine version is based on the growth inhibition of *Skeletonema costatum* and *Phaeodactylum tricornutum* algae. The freshwater test has been carried out in erlenmeyer flasks with test volumes of tens of millilitres and growth inhibition (i.e. cell number) has been calculated using a particle counter. However, small-scale testing using microtiter plates for cultivation and plate readers for effect detection have gained more attention lately.

Small crustaceans from *Daphnia* genus have reportedly been used in ecotoxicological tests as early as in 1929 and are still important test species, although their relevance to ecological risk assessment has gained some critique (Breitholtz *et al.*, 2006). Daphnids or water fleas (algae feeders) have been considered to be good test organisms due to their quite short life-cycle and their opaque outer layer that enables, for example, the observation of heart beating. Acute toxicity towards *Daphnia magna* is measured as immobility of the organisms in the test vessel after 24 and 48 hours exposure time (ISO 6341, 1996; OECD, 2004). In the 3-week long-term test the number of surviving parents, as well as their capability to produce healthy offspring, are used as endpoint (ISO 10706, 2000; OECD, 2008). Behaviour and presence of males are other endpoints used in daphnid test. *Ceriodaphnia dubia* is commonly used for ecotoxicological assessments especially in the USA according to the standard of ASTM International (originally American Society for Testing and Materials). Different species and strains respond varyingly to exposure, which makes the comparison of test results obtained in different studies somewhat difficult (Isidori *et al.*, 2006; Oda *et al.*, 2007). Also the test medium can have an effect on the test results. It has been shown that *D. magna* immobility tests using test medium with EDTA may result in EC50 values of one order of magnitude higher when compared to EDTA-free media (Guilhermino *et al.*, 1997). The presence of EDTA is especially important in the case of metals. The use of daphnids has been restricted mostly to the above mentioned types of studies. However, especially recently the use of daphnids for xenobiotics bioaccumulation and metabolism studies has been growing (e.g. Baldwin *et al.*, 1997; Akkanen and Kukkonen, 2003; Ikenaka *et al.*, 2006; Guan and Wang, 2006; Nakari and Huhtala, 2008).

Fish have been considered to be very important for ecotoxicological assessment, for example, due to their linking role in the food web between the aquatic environment and humans. A number of fish species have been used for ecotoxicological studies. One of the most popular laboratory test species in Europe is zebra fish (*Danio rerio*) due to its relatively small size and

short life-cycle. Methods to determine acute lethal toxicity of substances to zebra fish using static, semi-static and flow-through methods, as well as to assess acute toxicity to its eggs, have also been standardised. Also other fish tests have been standardised by ISO. Similarly, the OECD has harmonized a short term acute fish toxicity test used for chemical testing (OECD, 1992). The OECD's fish test guidelines cover also the toxicity tests on early life stages (duration 1-3 months) and embryo stages, as well as the growth test for juvenile fish. More detailed information on the effects on fish caused by the exposure can be gained when certain biochemical or genetical parameters (biomarkers) are studied from the exposed fish. Biomarkers often used in fish tests include the induction of vitellogenin production (egg yolk precursor normally absent in males) and induction of cytochrome P4501A and enzyme activity (metabolism of xenobiotics). Other common biomarkers are metallothionein (heavy metal exposure), acetylcholin esterase (organophosphate exposure) and glutathione S-transferase (oxidative stress). Fish tests, including also biomarker studies, have been applied especially for wastewater effluents, to measure exposure and effects revealing various types of harmful compounds during the past decades. Recently, the endocrine disrupting potential of effluents has been in the core of these studies.

### 1.1.3 Small-scale tests

Test procedures performed on microplates, or performed otherwise in a small and convenient manner, can be considered small-scale tests, although the original definition included only specific effects in unicellular organisms exposed to liquid samples (Blaise, 1998). Small-scale tests can be at least partially automated and many test methods, including required test materials and instrumentation, are commercially available today. These methods can also be considered to be more environmental friendly, as smaller reagent volumes and cultivation facilities are needed. Small-scale tests can utilize whole organisms or they can be based, for example, on cell-lines or sub-cellular components. Often there are specific biological interactions that are studied, and easily detectable reporters, for example luminescence, are used for detecting the response. Methods that are based on specific interactions give information on the presence of certain type of pollutants rapidly. This, however, means that a number of methods are needed in order to screen for varied types of adverse effects.

Some small-scale methods are introduced here. The methods have been divided based on the type of test organism (or system). Effect based division has been used in tables 1 and 2, which provide lists of small-scale methods for measuring estrogenicity and genotoxicity, respectively.

*Algae*
The algal microplate methods have proven to be a suitable alternative to the traditional flask method (Rojickova *et al.*, 1998; Eisentraeger *et al.*, 2003; Pavlic *et al.*, 2006). Several approaches for performing the freshwater algae test in a small scale exist and no uniform methodology has been developed yet (Arensberg *et al.*, 1995; Mayer *et al.*, 1997; Geis *et al.*, 2000; Horvatic *et al.*, 2007; Paixao *et al.*, 2008). However, in some countries algal microplate methods have even become national standards and also the latest ISO standard includes guidance on wastewater screening on microplates (ISO 8692, 2004). Applications of microplate methods on marine algal assays have been reported less (Blaise *et al.*, 1998). In microplate applications indirect methods (e.g. fluorescence, turbidity) for assessing algal growth inhibition are favoured. Fluorometric measurement is based on the fluorescent properties of algal chlorophyll and is considered more suitable than turbidity measurement, which can be affected by

turbid test samples (ISO 8692, 2004). Alternatively the chlorophyll may be extracted and the fluorescence measured after that.

*Bacteria*

Bacteria are popular test organisms as they are easy and fast to cultivate and genetic engineering has made them even more desirable for researchers: Using GM (genetically modified) organisms the range of properties and effects that can be studied is endless. However, GM organisms are acting as surrogates bearing properties that are foreign to them and, thus, microbes' own cellular processes may affect the results.

The best known and most widely used bacterial test organism is the naturally luminescent marine bacteria *Vibrio fischeri* (former *Photobacterium phosphoreum*). This organism has been used for ecotoxicity testing already in the late 1970's. The biochemical mechanism behind the luminescent property is explained in Figure 2. Due to the wide range of possible interference points, light production is thought to be a measure of the overall well-being of the bacteria. The inhibition of light production can be due to, for example, compounds that affect cell respiration, electron transport systems, ATP generation and protein or lipid synthesis. Of course also compounds that affect the cell's integrity and especially membrane function have an effect on *in vivo* luminescence.

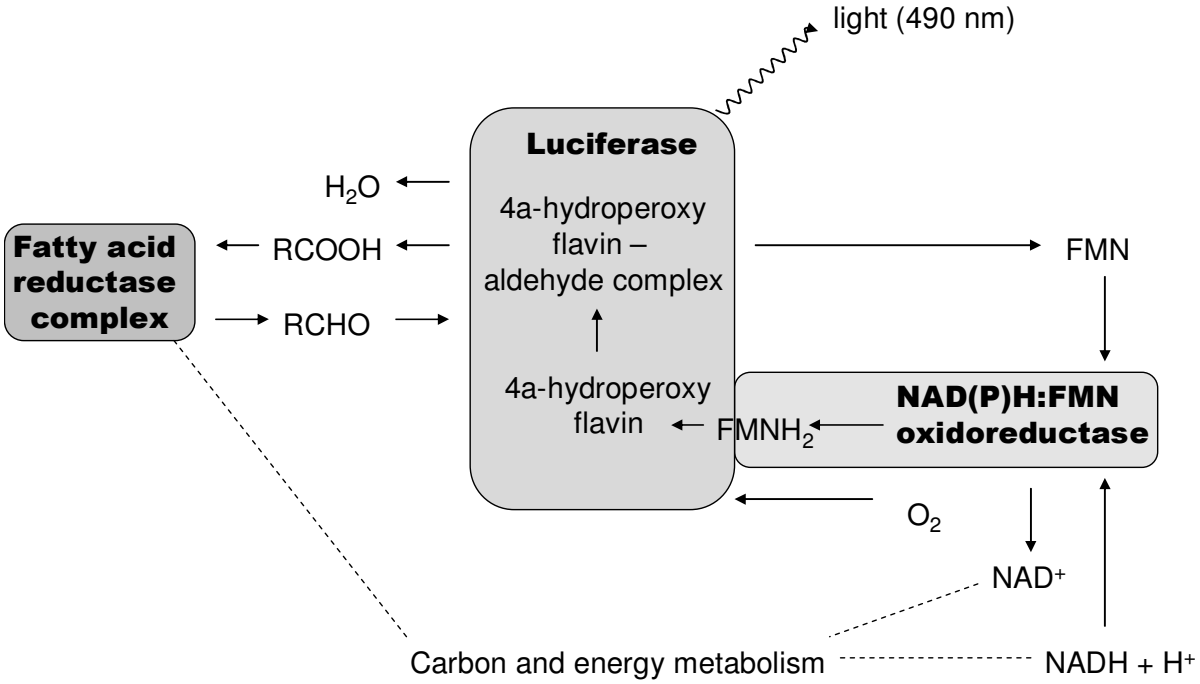



**Figure 2.** A simplified model of light production by *V. fischeri* bacteria with linkages to common bacterial carbon and energy metabolism (dashed lines). Reduced FMN is bound to luciferase forming with the other substrates an activated complex, which produces light upon decomposition. (Figure based on Meighen, 1991 and Jeffers and Tu, 2001).

The traditional acute luminescent bacteria test uses the inhibition of luminescence as an endpoint (e.g. ISO 11348-3, 2007) and test kits for this type of testing have been commercially available already for decades. Examples of test kits are Microtox® (Microbics Corporation, Carlsbad, USA) and Biotox® (Aboatox, Turku, Finland). Newer applications of the luminescent *V. fischeri* bacteria include the kinetic method (i.e. flash method) (Lappalainen *et al.*,

1999), growth inhibition method (Schmitz *et al.*, 1998; Gellert, 1999; Gellert *et al.*, 1999) and the mutation method using a dark mutant strain (Bulich, 1992). These whole organism tests are quite convenient to carry out and microplate applications do also exist. Recently, for example, a microplate version of the flash method was described and used for assessing nanoparticles (Mortimer *et al.* 2008). The easiness of measuring luminescence has triggered the development of other test systems that utilize the same detection method, i.e. *lux* genes from *V. fischeri* have been inserted into GMOs as reporter genes for certain cellular reactions.

*Salmonella typhimurium* is another bacterial species that has been extensively used in ecotoxicology, especially for revealing mutagenic potential. The traditional Ames test, using backmutation of $His^-$ strains on histidine deficient agar plates to discover mutagenic compounds, was invented already in the 1970's (Ames *et al.*, 1975). Different strains and applications of the Ames test have since been developed and used. The Ames test has been recently standardized by ISO (ISO 16240, 2005), but the traditional Ames test on plates is quite laborious and time consuming for monitoring purposes with the 2-3 days incubation time and the several different test strains used. Miniaturization of its liquid version (fluctuation test) is an approach to make the test more convenient. *S. typhimurium* has been used for detecting other types of genotoxic effects as well. Several assays based on the induction of SOS response (i.e. induction of the DNA repair system including SOS genes) and reporter genes in genetically modified *S. typhimurium* strains due to genome damage have been developed and are performed in small-scale. For example, in the Vitotox® assay the cascade of DNA repair mechanisms starts to function upon DNA damage, i.e. the transcription of the SOS genes is induced. Simultaneously also the transcription of *lux* genes that originate from *V. fischeri* are transcribed and the bacteria become luminescent (van der Lelie *et al.*, 1997; Verschaeve *et al.*, 1999). In the standardized umu-test the activation of SOS genes is detected by the infusion of *lac* genes, i.e. their activation results in production of β-galactosidase, which activity can be measured spectrophotometrically (ISO 13829, 2000). A complete automation of this method has been presented recently (Brinkmann and Eisentraeger, 2008).

Genetically modified *Escherichia coli* bacteria have been used in various applications in ecotoxicological studies. SOS Chromotest is a *E. coli* based genotoxicity test that is quite similar to the umu-test. *E. coli* has been used also for detecting metal toxicity. Metal specific sensor strains are used to detect bioavailable metals. MetPlate™ is a commercial application, based on the inhibition of β-galactosidase activity due to heavy metals (Bitton *et al.*, 1994). In addition, numerous metal specific biosensor constructions, also using other bacteria than *E. coli*, have been created and used especially in soil studies (e.g. Tauriainen *et al.*, 2000; Ivask *et al.*, 2002).

*Pseudomonas putida*, the ubiquitous gram-negative bacteria, has been used to study effects on bacterial growth. This standardised test system monitors the inhibition of bacterial cell multiplication using turbidity measurement and can be performed on multiwell plates (ISO 10712, 1995). The test duration is 16 hours. Recently, a high throughput screening system for the *P. putida* growth inhibition test has been developed (Spiller *et al.*, 2006). This 6 hour test is based on the use of conductometric sensors on 1536 well microtiterplate.

Respirometric methods have been used to assess inhibiting effects of samples on bacteria especially to protect the biological processes of wastewater treatment plants (WWTP) and have been used for studying both the inhibition of nitrifying bacteria and inhibition of respiration of heterotrophic bacteria, as reviewed by (Ren, 2004). Sludge bacteria, as well as pure cultures of *Nitrobacter* and *Nitrosomonas* have been used to study the inhibition of nitrification. For

example in Sweden even 60 % of the studied WWTPs were reported to receive wastewaters that inhibit nitrification (Jönsson *et al.*, 2000; Grunditz and Dalhammar, 2001).

*Yeasts*

In yeast cells the combination of easiness of culturing (similar to bacterial cultures) and the eukaryotic cell composition (similar to human cells) makes them excellent and interesting test organisms. Indeed, huge amounts of test methodologies utilizing yeast cells have been invented. Yeast cell constructions have been used for assessing, for example, growth inhibition, genotoxicity and hormonal effects. Genetical engineering has made it possible to introduce desired functions (e.g. of human origin) into yeasts, so that effect studies can be carried out in an easy – and ethically acceptable – manner. Yeasts have been important test organisms especially for the clinical sector, but they are suitable also for ecotoxicological purposes.

Recently, especially different types of *Saccharomyces cerevisiae* -based genotoxicity assays have been invented (Walmsley *et al.*, 1997; Lichtenberg-Fraté *et al.*, 2003; Cahill *et al.*, 2004; Benton *et al.*, 2007; Schafer *et al.*, 2008). One of these genotoxicity methods uses green fluorescent protein (GFP) fused to the *RAD54* promoter that is activated upon DNA damage as a reporter. This system may be considered to be more accurate than the SOS response-based genotoxicity methods, which could be activated also due to other stressors than just DNA damage (Cahill *et al.*, 2004). Test kits for this test are also commercially available (GreenScreen®, Gentronix Ltd). This method has been applied, for example, to study the effects of pharmaceuticals and different types of industrial effluents (Schmitt *et al.*, 2005; Keenan *et al.*, 2007; Zounková *et al.*, 2007).

Probably most attention has been paid on estrogen receptor specific interactions of substances in genetically modified yeasts. The human estrogen receptor (ER) is the most studied receptor in recombinant *S. cerevisiae*. Different types of human ER applications have emerged, but the concept that utilises the β-galactosidase as a reporter gene for ligand binding with estrogen receptor has been used most often (Routledge and Sumpter, 1996). Its drawback is the length of the incubation time, as it takes 3 days before the results are ready. Therefore, other faster methods have been developed. One example is the method in which the detection of estrogenic effect is based on luminescence and the results are ready within hours (Leskinen *et al.*, 2003). ER yeast tests have been successfully applied to chemicals (e.g. alkylphenols, phytosterol esters), as well as environmental samples (e.g. effluents, sediments, surface waters) (Routledge and Sumpter, 1997; Baker *et al.*, 1999; García-Reyero *et al.*, 2001; Céspedes *et al.*, 2004; Kim *et al.*, 2004; Pawlowski *et al.*, 2004). Yeast-based tests have been developed also for human androgen and progesterone receptors (e.g. Gaido *et al.*, 1997; Michelini *et al.*, 2005). For aquatic environmental samples receptors originating from other species than humans could be more preferable and, for example, Petit *et al.* (1995) have constructed a recombinant yeast with rainbow trout estrogen receptor.

*Cell-lines and isolated cells*

Cultured cell-lines have also been used for ecotoxicological studies, although they are more familiar to toxicological assessments. Among the most used are the human MCF-7 breast cancer cells. They have been used for screening of estrogenic effects using a test called E-screen based on the proliferation rate of the cancer cells (Soto *et al.*, 1995). Another method based on the human breast cancer cell is ER-CALUX (estrogen receptor chemical-activated luciferase gene expression), in which an ER regulated luciferase reporter gene is set in the T47D cells (Legler *et al.*, 1999). However, cell lines of animal origin can be considered more useful for ecotoxicological assessments. Such have successfully been derived for several fish

species and cell types (e.g. hepatonema cells from topminnow and gonad cell line RTG-2 from rainbow trout) and have been used especially for studying cytotoxicity, induction of cytochrome P4501A and vitellogenesis, as reviewed by Fent (2001). GM rat hepatoma H4IIE cell line, that carries firefly luciferase gene measures binding of compounds to arylhydrocarbon receptor. This microplate test system has been marketed under the name DR-CALUX (dioxin responsive chemical-activated luciferase gene expression) and is used to assess exposure to dioxin or dioxin-like compounds (BioDetection Systems, The Netherlands).

Similarly, newly isolated cells can be used for ecotoxicological testing. With isolated cells the culturing of cell-lines is avoided, but the isolation of cells is also labour intensive and needs special skills. Cells may loose some of their properties in continuous cultivation, which, as well as the fact that cell lines are usually of cancer cell origin, can be considered as disadvantages of cell lines.

*Sub-cellular particles*
Sub-mitochondrial particles (SMP) isolated from beef heart have shown to be suitable for ecotoxicological studies. Mitochondrial membranes contain a lot of enzymes, and changes in their functions can be exploited in evaluating toxic effects. Inhibition of electron transport on the inner mitochondrial membrane has proven to be a sensitive, rapid and reproducible method to screen for toxicity (Knobeloch *et al.*, 1994; Gustavson *et al.*, 1998; Read *et al.*, 1998; Gustavson *et al.*, 2000; Doherty and Gustavson, 2002). Both reverse (RET) and forward (ETr) electron transport along the transport chain have been utilized for toxicity assays. This *in vitro* methodology has been applied successfully to a wide variety of samples, such as chemicals, wastewaters and extracts of environmental samples (Argese *et al.*, 1995; da Silva *et al.*, 1998; Juvonen *et al.*, 2000; Schultz *et al.*, 2002; Schultz *et al.*, 2004; Argese *et al.*, 2005). The lack of most cellular functions, for example uptake and metabolisms of compounds, is a disadvantage of *in vitro* methods. They are, however, especially valuable as screening methods and for studying the mechanism of action.

In case of RET, the excess energy brought into the test system as ATP is resulting in reverse transport of electrons. This reverse transport of electrons causes finally an increase in the NADH concentration, which can be detected by spectrophotometer as an increase of absorbance at wavelength 340 nm. Disturbance of the electron flow by a toxicant can be detected as a decrease in the enzymatic formation of NADH. A more detailed view of the biochemical mechanisms is presented in Figure 3. In the ETr method the electron flow is forward, i.e. into the direction that generates ATP and does not consume it. In these cases the inhibition of electron flow lowers the NADH consumption in the test system.

Sub-cellular particles can also be used for pre-treatment of samples prior to actual ecotoxicological assays. Such an approach is often used in genotoxicity testing that utilise microbes as test organisms. Microbes do not possess similar metabolic capabilities as higher organisms do. Thus, sub-cellular fractions containing xenobiotic metabolizing enzymes (cytochrome P4501A) are used to find out about the possible impact of metabolism on the biological effect. This sub-cellular fraction (also called S9) is often obtained from rats that have been exposed to polyaromatic hydrocarbons, but for aquatic ecotoxicological studies also fish S9 fractions have been used.

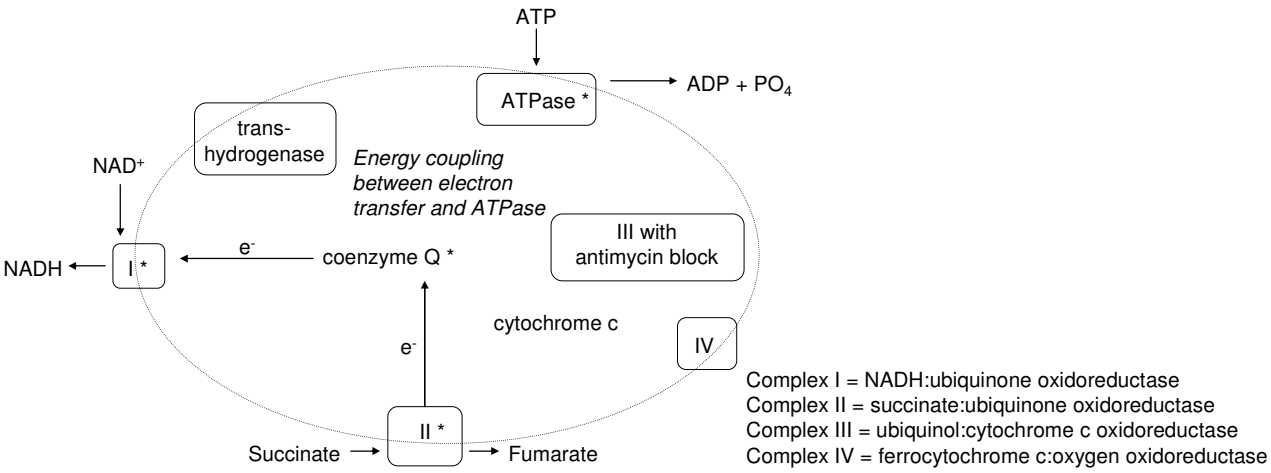



**Figure 3.** Components of electron transport chain on mitochondrial inner membrane and those that are active in RET are marked with * (modified from Knobeloch *et al.*, 1990 and Read *et al.*, 1998).

**Table 1.** Examples of small-scale methods for detecting estrogenic effects.

| Test name | Organism/ cell type | Species/cells | Detection | Reference |
|---|---|---|---|---|
| E-Screen | Human breast cancer cells | MCF-7 cell line | Proliferation rate of the human cancer cells is calculated from the number of nuclei after lysing the cells. | Soto *et al.*, 1995 |
| YES | Yeast | *Saccharomyces cerevisiae* with human ERα | Activation of human ER by a ligand causes the transcription of *lac-Z* genes producing β-galactosidase. Activity of β-galactosidase is detected by spectrofluorometer. | Routledge and Sumpter, 1996 |
| ER yeast | Yeast | *Saccharomyces cerevisiae* with human ERα | Activation of human ERα by a ligand causes the transcription of *luc* genes and formation of luciferase. Activity of luciferase is detected using luminometer after the addition of D-luciferin. | Leskinen *et al.*, 2003 |
| ER yeast | Yeast | *Saccharomyces cerevisiae*with the hormone binding domain of human ER | Binding of hormone causes the ER binding domain – GAL4-DNA binding domain complex to activate the β-galactosidase genes. Activity of β-galactosidase is detected by spectrofluorometer. | Louvion *et al.*, 1993 |
| ER-CALUX | Human breast cancer cells | T47D cell line | Activation of human ER by a ligand causes the transcription of *luc* genes and formation of luciferase. The cells are lysed prior to luciferin addition and luminescence measurement. | Legler *et al.*, 1999 |

**Table 2.** Some small-scale test systems for detecting genetic disturbance.

| Test name | Organism/ cell type | Species/cells | Endpoint | Detection | Test duration | Reference |
|---|---|---|---|---|---|---|
| Ames fluctuation test (e.g. muta-chromo-plate) | Bacteria | *Salmonella typhimurium* TA 98 and TA 100 (other strains are possible) | Mutagenicity | His revertants cause acidification, which is detected as a colour change from red to yellow. Yellow wells are counted e.g. using a spectrophotometer | 72-120 h | e.g. Rao and Lifshitz, 1995 |
| Mutatox® | Bacteria | *Vibrio fischeri* (dark mutant) | Mutagenicity | Luminescence measurement after backmutations | 18 h | Bulich, 1992 |
| *Umu*C | Bacteria | *Salmonella typhimurium* TA1535/pSK1002 | Genotoxicity | Activation of *umuC* operon upon genotoxic lesions in DNA. This can be detected as increased β-galactosidase activity using a spectrophotometer | 4 h | ISO 13829, 2000 |
| Vitotox® | Bacteria | *Salmonella typhimurium* TA104 recN2-4 and TA104pr1 | Genotoxicity | Induction of SOS response leads to the derepression of *lux* genes and can be measured with a luminometer | 4 h | Verschaeve *et al.*, 1999; van der Lelie *et al.*, 1997 |
| SOS Chromotest | Bacteria | *Escherichia coli* K-12 | Genotoxicity | Inhibition of cell division causes the expression of SOS function coded by *sfiA* that activates *lacZ* operon. This can be detected using a spectrophotometer. | 2 h | Quillardet *et al.*, 1982; Xu *et al.*, 1989 |
| Yeast test with RAD54/ Green-Screen® | Yeast | *Saccharomyces cerevisiae* | Genotoxicity | Detection of the transcriptional activation of GFP by DNA damage inducible *RAD54* promoter using fluorometer | 16 h | e.g. Schmitt *et al.*, 2004; Cahill *et al.*, 2004; Lichtenberg-Fraté *et al.*, 2003 |
| Comet assay / Single cell gel electrophoresis | Eukaryotic cells (*in vivo* and *in vitro*) | | Genotoxicity | Detection of negatively charged single and double DNA strand breaks using microgel electrophoresis, fluorescent dye and image analysis | Not relevant | Singh *et al.*, 1988 |
| Micronucleus micromethod (*in vitro*) | Eukaryotic cells | e.g. L5178Y mouse lymphoma cells | Genotoxicity | Exposure on microplates followed by microscopical detection of micronucleated cells on stained slides | 24 h | Nesslany and Marzin, 1999 |

### 1.1.4 Fingerprints and micro-scale screening tools

Biochips and microarrays are small devices, usually glass slides, containing numerous cDNA (complementary DNA) fragments, which can be used to detect RNA (ribonucleic acid) extracted and turned into cDNA from exposed organisms, tissues or cells. They have been developed and applied especially for toxicological uses, such as, testing chemicals, cosmetics and pharmaceuticals, simulating mainly human exposure. However, similar approaches could be suitable also for environmental evaluations. And indeed, ecotoxicogenomics, meaning the exploitation of the familiar tools from genomics to ecotoxicological purposes, has gained increasing attention lately. In addition to the study of expression levels of messenger RNA (i.e. transcriptomics), studies on protein (proteomics) and metabolite (metabolomics) levels and patterns may provide tools for ecotoxicological assessment in the future.

Classical biomarker studies (mentioned also in chapter 1.1.1.) have been applied singly to assess exposure and effects. However, development of microarray techniques has enabled the examination of multiple gene expressions simultaneously, for example in aquatic animals. Results from such studies give the possibility to create expression profiles, i.e. fingerprints that combine the information from multiple gene expressions into one concise database. Such a fingerprint approach has been proposed to be used also for test results from a battery of ecotoxicity tests. For example, in a test system containing several different types of bacterial species and strains (preferably even on one single microplate) the results can be combined into one ecotoxic fingerprint (Gabrielson *et al.*, 2003).

Microarrays have been applied recently to study exposure of potential toxicants to several aquatic organisms, such as rainbow trout and water flea (e.g. Koskinen *et al.*, 2004; Hook *et al.*, 2006; Finne *et al.*, 2007; Poynton *et al.*, 2007; Mos *et al.*, 2008). However, the development of cDNA microarrays is quite costly, time consuming and labour intensive. Thus it has been suggested that in stead of developing own microarrays for all species, heterologous microarrays that would suit closely related species should be invented (Kassahn, 2008). A salmonid microarray that contains cDNA from Atlantic salmon and rainbow trout has shown to be efficient in spotting rainbow trout cDNA. It has been applied for studying single substance and mixture effects in laboratory both *in vivo* and *in vitro*: Adult rainbow trout fishes, fry and hepatocytes have been used in the studies (Hook *et al.*, 2006; Finne *et al.*, 2007; Mos *et al.*, 2008). The microarray results seem promising and, for example, the use of hemoglobins, prostaglandins, cytochromes and glutathione-S-transferases have been suggested as biomarkers in microarray-based risk assessments (Mos *et al.*, 2008).

### 1.1.5 Biodegradation tests

Degradation of chemicals and, for example, wastewater constituents by micro-organisms can be studied with so-called biodegradation tests, of which many are standardised. Biodegradation means the transformation of the substance studied due to the activity of microbes, i.e. the original substance disappears because the microbes turn it into other compounds and into biomass. Mineralization (i.e. ultimate biodegradation) means that the substance under scrutiny is turned into inorganic carbon dioxide ($CO_2$), water and biomass. Taking this into account, biodegradation can be measured in many different ways, for example, as the concentration of original substance, evolved $CO_2$ or dissolved organic carbon (DOC). The results are often expressed as biodegradation percentages, but also other endpoints are used (e.g. biodegrada-

tion rate). A range of methods for assessing biodegradability in different (environmental) conditions and for different purposes do exist, and some of them are presented below.

Measurement of biological oxygen demand (BOD) is the best known and most widely applied biodegradation method. It is often used for assessing wastewaters together with physico-chemical parameters. BOD gives a measure of oxygen consumption in relation to the biodegradation of organic substances. Inoculum for degraders is usually obtained from the activated sludge of a biological wastewater treatment plant (WWTP). The incubation time in BOD studies are 5 or 7 days and the results are calculated by comparing the oxygen amount at the end of the test to the situation at the beginning of the test. This test with relatively short duration is used to control the quality of wastewaters to prevent discharges with high oxygen demand and consequently oxygen depletion in receiving waters, rather than to gain information on biodegradation rates or mineralization.

The $CO_2$ headspace test (ISO 14593, 1999; OECD, 2006), on the other hand, is designed to assess the mineralization of substances. Also in this test, as in many of the aerobic biodegradation tests, the inoculum is obtained from activated sludge. In some biodegradation methods the inoculum is allowed to acclimatize to the test substance using pre-exposure, but in this headspace test the microbes are used "as they come". However, it is possible to influence the biodegradation results by the selection of inoculum source: Inocula are naturally acclimatized to substances that are common at their WWTP. In the headspace test the studied substance is the sole carbon source (20 mgC/l) for the entire test duration (28 d), meaning that the microbes capable of using it as primary substrate will grow exponentially. By measuring the evolved $CO_2$ and comparing it to the theoretical $CO_2$ production biodegradation percentages can be calculated. Also biodegradation rates (1/day) can be estimated from the headspace test, although their relevance to chemical risk assessment has been criticized and questioned, because they differ so much from those obtained under environmental conditions (Ingerslev *et al.*, 1998; Ahtiainen *et al.*, 2003; ISO/TR 15462, 2006).

The shake-flask method (ISO 14592-1, 2002; OECD, 2004) simulates biodegradation in the environment using surface water as test medium and as a source for degraders. The test sample concentrations are much lower than in most other biodegradation test systems in order to be more consistent with the actual environmental conditions. In this approach, however, the amount of evolved $CO_2$ or remaining DOC is too small to be measured. Thus, substance-specific methods to determine the biodegradation or use of radiolabelled substances are needed.

## 1.2 Wastewater effluents

### 1.2.1 Wastewater characteristics

Wastewaters are divided into three categories based on their origin: domestic (discharges from residential settlements and services originating from human metabolism and household activities), industrial (discharges from industry premises) and urban (domestic wastewater with possible industrial and run-off rain water) (EC, 1991). Urban wastewaters are usually treated at municipal wastewater treatment plants (WWTPs), at least in Finland, and thus the effluents are often called municipal effluents. Large industrial plants do process their wastewaters usually in their own WWTPs, but wastewaters from smaller units are typically treated at municipal WWTPs together with domestic wastewaters.

In Finland there are approximately 560 WWTPs treating urban wastewaters in population centers with over 50 inhabitants (Santala *et al.*, 2006). This results in 500 million cubic metres of wastewater to be treated each year. In year 2004 the approximate loads from municipal WWTPs were for phosphorus 200 tons, for nitrogen 12200 tons and for organics ($BOD_7$) 5300 tons. Urban wastewaters could be considered quite uniform in composition as they originate from basic human activities. However, regional and seasonal differences are common and the wastewaters originating from small to medium-size industrial activities obviously give their specific character to the wastewaters treated at municipal WWTPs and the effluents they discharge. Also the unit processes and operations chosen to treat the wastewaters certainly affect the composition of the final effluent. In case of urban wastewaters, changes in the wastewater character are often related to heavy rain falls, which may dilute the wastewater, but also increase the wastewater volumes momentarily to levels in excess of the plants treatment capacity.

Industrial effluents, on the other hand, do vary much more in their composition as every industrial sector has its own specific wastewater characteristics. For example pulp and paper mill effluents are rich in organic matter and nutrients: In year 2006 effluents originating from pulp and paper industry constituted 92, 79 and 86 % of the total $BOD_7$, nitrogen and phosphorus loads, respectively, although their contribution to the total industrial effluent volume was only 65 % (Finnish Environment Institutes statistics). Characteristic for industrial wastewaters is also that within one industrial plant there may be many wastewater streams with distinctly different properties and they may be produced periodically depending on the type of the process. In addition, industrial plant shutdowns are quite regular, which in turn affects the WWTP and its operations.

### 1.2.2 Organic compounds in wastewater treatment

Modern WWTPs commonly consist of physical, chemical and biological treatment processes. Physical treatments (e.g. grates and screens) are used to separate coarse particles out of the wastewater. Particles may be in the wastewater already when they come to the WWTP or new particular mass may be formed in the treatment processes: For example in chemical treatment phosphorous can be precipitated using metal salts.

Biological treatment processes (also called secondary treatment) are targeted especially at attacking organic compounds. Both aerobic and anaerobic treatments are possible and they are often combined consecutively together with nitrogen removal in order to obtain the best possible treatment efficiency. Activated sludge processes are the most applied biological treatment processes in Finland. They are based on aerobic micro-organisms in an aerated basin degrading and assimilating as much of the wastewater compounds as possible under the prevailing conditions. The activated sludge process is optimized by adjusting, for example, the hydraulic retention time (HRT) and sludge loading. Usually longer HRTs favour better treatment results. After activated sludge treatment the microbes containing sludge is usually separated from the treated wastewater by settling in a clarifier.

The recalcitrant compounds that are not degraded or are only slightly modified by the microbes may attach to the sludge, enter the next treatment step or may be finally discharged into the receiving waterway. Examples of compounds with incomplete biodegradation at WWTPs are some hormones (e.g. estrone), pharmaceuticals (e.g. metoprolol), surfactants

(e.g. alkylphenol ethoxylates, APEs) and natural polymers (e.g. lignin) (e.g. Fujita *et al.*, 2000; Helmreich *et al.*, 2001; Johnson *et al.*, 2005; Vieno *et al.*, 2007). From nonylphenol ethoxylates (NPEs), the most common APEs, recalcitrant metabolites such as nonylphenol di- and triethoxylates (with and without carboxylation) and nonylphenol are often formed rapidly in biological degradation processes (e.g. Di Corcia *et al.*, 1998; Staples *et al.*, 2001; Langford *et al.*, 2005). In case of high molecular weight (HMW) lignin, bacterial attack is very limited. However, lignin removal by secondary treatment is possible, but its efficiency has been related mostly to adsorption onto sludge (Helmreich *et al.*, 2001; Pokhrel and Viraraghavan, 2004).

### 1.2.3 Controlling and monitoring wastewater effluents

Treated wastewaters that are discharged to the receiving waterway still include a wide variety of substances despite the extensive treatment processes. For example Kirk *et al.* (2002) measured the removal of estrogenicity by various WWTPs and showed that even 20 % of the endocrine disrupting potential passes trough WWTPs with secondary treatment.

The fate and effects of the discharged substances depend on their physico-chemical properties and on the properties of the receiving waterway. In order to protect the environment, consent conditions for the wastewater effluents to be discharged are set based on risk assessments. Limits set by authorities for discharges typically include parameters such as nitrogen, phosphorous, suspended solids and BOD measuring predominantly the physico-chemical quality of the effluents.

A recent major water quality related achievement within the EU is the Water Framework Directive (WFD), which aims at good water quality of water resources measured by ecological and chemical parameters (EC, 2000). Within WFD the good ecological status is based on the composition and abundance of certain taxa in the waterways. Also the 33 priority substances, or substance groups, that are not allowed to be discharged in the EU form an important part of the good water quality objectives of the WFD. In addition, it has recently been suggested that biomarkers could be valuable tools for WFD related investigational and operational monitoring (Allan *et al.*, 2006).

By monitoring the biological status of the waterways it is possible to observe the disturbances the environment has confronted. In this case, however, the harm has already been done and remedial processes to restore good water quality may consume time and money – or even be mission impossible. Another – or supplemental – approach to ensure the good ecological status of the waterways is to control the discharges of toxic pollutants by controlling and monitoring the quality of wastewater effluents by biological methods. Indeed, in some countries bioassays have been incorporated into discharge consent conditions as set by the national legislation or environmental authority (Power and Boumphrey, 2004). In Finland, the authorizing body may require ecotoxicological assessment of the effluents, but neither national guidance nor regulations on threshold limits are available. Thus, the policy varies from country to country when it comes to utilising biological methods in monitoring effluent quality. The USA and Germany are examples of countries, in which bioassays form an important part of the discharge consent conditions and thus wastewater effluents are monitored routinely using ecotoxicological methods.

In Germany provisions for the discharge of wastewaters of municipal and industrial origin are laid down in the national Waste Water Ordinance (BMU, 2004). The requirements are sector-based for both existing and new discharges comprising altogether 53 different types of wastewaters. In addition to physico-chemical requirements, it defines also biological parameters that are to be considered. Toxicity to fish eggs, daphnia, algae and luminescent bacteria form the basic battery of biological parameters, the fish egg toxicity test being stipulated for most of the wastewaters. In case of chemical industry effluents also the umu-test that measures genotoxicity is included. The discharge limits are based on selected effluent dilution levels that may not comprise marked toxicity.

In the USA the Environmental Protection Agency's (EPA) National Pollutant Discharge Elimination System (NPDES) regulates discharges to the water bodies. EPA recommends that whole effluent toxicity (WET) tests are used in NPDES permits together with chemical-based water quality criteria. WET test methods promulgated by EPA include acute and chronic test for freshwater, marine and estuarine species (e.g. algae *P. subcapitata*; daphnids *C. dubia* and *D. magna*; mysid *Mysidopsis bahia*; fishes *Pimephales promelas* and *Oncorhynchus mykiss*; sea urchin *Arbacia punctulata*). If a facility does not pass its toxicity-based discharge limits, a site-specific Toxicity Reduction Evaluation (TRE) may be carried out (U.S. EPA, 2001). It aims in a step-wise process to identify the compounds responsible for the effluent toxicity, to isolate the source of toxicity and to evaluate the effectiveness of the control options. Finally, toxicity reduction is confirmed after the control measures have been implemented.

## 1.3 Fractionation methods for effluents

### 1.3.1 Purpose of fractionation

In case of whole-effluents (or other environmental samples) that show adverse biological effects, it is time-consuming and maybe even impossible to try to find the cause of toxicity just by chemical analyses. Thus, fractionation of the samples into smaller subunits is used to track the cause of toxicity: Physico-chemical fractionation methods give hints on the properties of the toxicant(s) and following those the cause of toxicity may be revealed. In addition, fractionation may sometimes reveal toxicity that is not detected, or is less, in whole-effluent samples due to antagonism. This means that the combined effect of harmful compounds is less than their separate additive effects.

The overall chemical analysis of fractions as directed by the outcome of the ecotoxicity tests is today often called effects-directed analysis (EDA). Such fractionations have been applied for tracking harmful components from a wide variety of environmental samples, such as sediments (Carr *et al.*, 2001), effluents (Burkhard and Jenson, 1993; Yang *et al.*, 1999; Mount and Hockett, 2000; Grung *et al.*, 2007), groundwater (Gustavson *et al.*, 2000) and surface water (Reineke *et al.*, 2002). The methods are somewhat case- and country-specific, but the mostly applied and most formal concept is the Toxicity Identification Evaluation (TIE) published by the U.S. Environmental Protection Agency (U.S. EPA) in the 1980's. TIE was originally developed for acutely toxic effluents not meeting their consent conditions. Later U.S. EPA developed TIE procedures also for sediments and marine environments (U.S. EPA, 1996, 2007). Its variations and some other fractionation methods are presented in the following sub-chapters.

### 1.3.2 Toxicity identification evaluation

The U.S. EPA TIE approach for wastewater effluents is divided into three phases (Phases I-III). Phase I aims at characterizing the physico-chemical properties of the effluent constituents that are causing acute toxicity (U.S. EPA, 1991). The principle of Phase I is that effluent samples are manipulated by specific physical and chemical means and the samples are tested with biotests (mainly *Ceriodaphnia* or *Daphnia* species) before and after these manipulations. A schematic presentation of the procedure can be found in Pessala *et al.* (2004). The TIE procedure was originally used for tracking toxicants that cause acute effects in whole organism tests, but later also chronic toxicity was included.

The whole battery of manipulations included in Phase I is described in Table 3, as well as the purpose of each step. Most of the manipulations are targeted at inorganic toxicants, whereas organic toxicants are mainly affected by pH manipulations and subsequent aeration, filtration and SPE treatments. Information gained in Phase I can be used as a starting point for the toxicant identification or, if it is not necessary to identify the toxicants, merely for developing toxicity removing processes.

**Table 3.** Fractionation of effluents according to the U.S. EPA TIE Phase I scheme.

| Manipulation | Purpose of the manipulation |
|---|---|
| Initial toxicity | Represents the "true" toxicity of the effluent samples before possible changes in the sample composition as it is measured immediately after sample arrival. |
| Baseline toxicity | Represents the "before manipulation" toxicity as it is measured simultaneously with the manipulated samples. |
| EDTA addition | Chelates certain cationic metals (e.g. copper and zinc). |
| $Na_2S_2O_3$ addition | Reduces compounds (e.g. chlorine dioxide) and chelates cationic metals (e.g. copper and cadmium). |
| pH gradient | Samples are tested at three different pH values (pH 6-9); Affects the dissociation and speciation of compounds (e.g. ammonia, pentachlorophenol and zinc). |
| pH adjustment (pH 3, pH i, pH 11) | In addition to the initial pH value (pH i) the sample is adjusted to two additional pH values, but readjusted to pH i before toxicity tests; Affects the solubility, volatility, polarity, stability and speciation of the compounds. |
| - Aeration | Changes or removes volatile (e.g. ammonia), sublatable (e.g. detergents) and oxidizable (e.g. $S^{2-}$) compounds. |
| - Filtration | Removes insoluble material (e.g. metal complexes, particles with sorbed compounds) |
| - SPE (C18 column) | Removes organic compounds and metal chelates that are relatively nonpolar at the particular pH (due to the pH limitations of C18 columns SPE is carried out at pH 3, pH i and pH 9). |

The toxicants that have been characterized in Phase I are specifically identified in Phase II. However, the Phase II identification procedure published by U.S. EPA (U.S. EPA, 1993a) covers only metals, ammonia, non-polar organics, surfactants and chlorine. To identify non-polar organic toxicants SPE columns are eluted with a methanol series (varied water-methanol ratios) and toxic eluates are further fractionated with HPLC and an attached fraction collector. Obtained fractions are tested for toxicity and the compounds in toxic fractions are identified using GC/MS.

The final step of TIE is Phase III, which aims at confirming that the correct cause, or causes, of toxicity have been identified and that no possible toxicant has been left out of consideration (U.S. EPA, 1993b). The type of confirmation steps (Table 4) needed for a certain effluent and toxicant are case-specific.

**Table 4**. Possible toxicant confirmation steps as proposed in U.S. EPA TIE Phase III.

| Confirmation step | Task of the confirmation step |
|---|---|
| Correlation approach | Shows if there is a relationship between the concentration of the suspected toxicant and effluent toxicity when several effluent samples are compared. |
| Symptom approach | Compares the symptoms that are caused by the effluent samples and suspect toxicants. |
| Species sensitivity approach | Compares the species sensitivity ratios of the effluent samples and suspect toxicant. |
| Spiking approach | Compares the proportional changes in toxicity when effluent samples are spiked with certain amount of suspect toxicant. |
| Mass balance approach | Compares toxicities of non-toxic fractions (toxicity has been removed with SPE), toxicity restored fractions (each toxic fraction has been added to non-toxic fraction) and all-fractions. |
| Deletion approach | Compares toxic industrial effluent samples to samples taken after use of the suspect toxicant has been stopped for a while. |

### 1.3.3 Other fractionation methods

Most of the fractionation methods developed to enhance the original TIE are targeted at the detection of organic toxicants, meaning mainly the SPE and HPLC process. Already in the TIE Phase II procedure several alternatives to C18 columns are mentioned (XAD-4, XAD-7, C8 bonded silica sorbent and C18 bonded silica particles formed into disks with an inert material), but only the C18 materials were found suitable by the authors (U.S. EPA, 1993a). However, more elaborated SPE systems have been developed and evaluated for EDA suitability since then.

Especially the new polymer based SPE materials, ethylvinylbenzene-divinylbenzene (EVB-DVB) and poly(styrene-divinylbenzene) (PS-DVB), have been considered potential for EDA. Their advantages are a high extraction capacity due to their high specific areas, and their suitability to extract polar compounds from aqueous solutions. Sequential solid phase extraction schemes based on multiple column types and eluents have been described by Fiehn and Jekel (1996) and Castillo *et al.* (1999). The approach proposed by Fiehn and Jekel (1996) is a four step procedure that enables also the separation of polar compounds with the new polymeric columns: First, an end-capped C18 column (pH 7) retains neutral hydrophobic compounds. Second, a PS-DVB column (pH 7) retains slightly polar compounds. Third, an EVB-DVB column (pH 4.5) retains compounds with mixed functionalities and acids. Fourth, an EVB-DVB column (pH 2.5) ensures that all acidic compounds are retained. This kind of sequential SPE has been used, for example, to study tannery wastewaters (Reemtsma *et al.*, 1999).

# 2 Objectives of the study

The overall aim of the study was to assess wastewater effluents with biological and chemical methods and to track and characterise their possible toxicants. In addition, the impact of biodegradation on the harmful properties of compounds appearing in wastewater effluents was assessed.

In Finland biological methods are not routinely used for assessing and controlling wastewater effluents, whilst, for example, in the USA schemes such as TRE/TIE have been used for evaluating and reducing the effluent toxicities for years. Thus, the study aimed at applying parts of these methods to Finnish wastewater effluents. The aim was also to further develop and enhance the methods for assessing wastewater effluents and their toxicants using small-scale bioassays.

The specific aims addressed in the five original publications were:

1. To screen municipal and industrial wastewaters using chemical analyses and a battery of small-scale bioassays (Article I)

2. To characterise the effluents and possible toxicants using a modified TIE Phase I scheme (Articles I and II)

3. To identify suspect toxicants from pulp and paper mill wastewater effluent (Article II)

4. To characterise chemical structures that could explain the toxicity of an identified toxicant by combining chemical, biological and statistical methods (Articles III and IV)

5. To evaluate the biodegradation of organic compounds appearing in wastewater effluents using biological and chemical assessment (Article V)

# 3 Materials and methods

## 3.1 Overview of materials and methods

Materials used in the study comprise effluent samples, a spiked reference effluent, laboratory-scale effluents and model compounds. Wastewater effluent samples (24-hour composite) from altogether nine different WWTPs were collected including three municipal and six industrial WWTP samples (**I**). Repeated samplings were conducted at two industrial WWTPs: two samples from a WWTP treating wastewaters from pharmaceuticals and enzyme production (**I**) and five samples from a WWTP treating mainly pulp and paper mill wastewaters (**I**, **II**). Laboratory-scale pulping and bleaching effluents were studied in articles **III** and **IV**. Model compounds were used for evaluating the modified TIE procedure (**I**), for assessing candidate toxicants (**II**, **III**) and for assessing biodegradation of toxicants (**V**).

A number of biological and chemical methods were used for assessing the wastewater effluents and the toxic components in this study. The combination of chemical and biological methods is essential in the tracking, characterisation and identification of toxic compounds, however, the main focus of the author was on the biological methods (especially microbiological methods) and the fractionation procedure. The methods used in this study are described in detail in the original publications. The modifications of the TIE Phase I method is described in the following sub-chapter and a summary of all methods used in the study can be found in Tables 5 and 6.

## 3.2 Modified TIE

The ecotoxicity tests used in the study were mainly small-scale tests with varied endpoints in contrast to the acute (and chronic) whole organism tests of the U.S. EPA TIE procedure. This assured that a wide range of possible responses could be recorded and it also speeded up the testing procedure as the test durations were in many cases only a few minutes or hours. The effluent fractionations were carried out according to the TIE Phase I procedure with the following modifications:

Aeration at three different pHs (pH 3, pH i and pH 11) was performed using nitrogen stream. Thus, volatilisation and sparging were the only potential ways for the toxic compounds to be removed and oxidizable compounds were thus not affected. pH gradient was achieved by adjusting the sample pH to 7-8,5 for RET and 6-8,5 for *V. fischeri* assays. In RET assay also the test buffer was adjusted to corresponding pH value to assure the stability of the pH gradient during the test. For the *V. fischeri* assay the test medium was adjusted roughly to the corresponding pH.

The SPE manipulations were carried out at pH 3, pH i and pH 11 using two successive poly(N-divinylpyrrolidone-divinylbenzene) columns (Oasis HLB, Waters). The columns have two-fold properties, i.e. polar and non-polar compounds can be retained. The columns tolerate higher pH values than the C18 columns (only up to pH 9) used in the original TIE procedure, thus enabling the same pH (pH 11) to be used as for other pH adjustment steps. The columns were preconditioned using 5 ml acetate and 5 ml of methanol followed by equilibration with pH adjusted water. The sample loading (100 ml) was conducted in two consecutive steps: First 20 ml of the sample was passed through the columns and the post column sample was

collected and tested. The rest of the sample (80 ml) was passed through the columns and the toxicity of this post-column sample was also measured. By comparing toxicities of these two post-column samples, the possible overloading of the columns could be controlled.

**Table 5.** Ecotoxicological and fractionation methods used in the original publications relating to this study.

| Test species or method | Test endpoint or aim of the method | Publication |
|---|---|---|
| ***Ecotoxicological methods*** | | |
| *Vibrio fischeri*<br>- traditional<br>- kinetic | Inhibition of luminescence<br>Measured in tubes after 30 minutes contact time<br>Measured on 96-well microplates; kinetic measurement during first 30 seconds followed by 5, 15 and 30 minute measurements | <br>I, II<br>III |
| Reverse electron transport | Inhibition of RET in submitochondrial particles measured as an increase in NADH content using spectrophotometer | I, II, III, IV, V |
| *Pseudokirchneriella subcapitata* | Inhibition of growth of green algae on microplates measured as fluorescence | I |
| *Salmonella typhimurium* | Genetic disturbance i.e. induction of SOS response in recombinant bacteria (Vitotox) measured as increasing luminescence | I |
| *Saccharomyces cerevisiae* | Induction of hER in recombinant yeast measured as increasing luminescence | V |
| Adenosine triphosphate | Enzymatic and luminometric assay of ATP content | V |
| Headspace test | Measurement of mineralisation as $CO_2$ or $CO_3^{2-}$ production | V |
| Vitellogenin | Induction of vitellogenin in freshly isolated rainbow trout hepatocytes measured using ELISA and microplate reader | I, III, V |
| Ethoxyresorufin-O-deethylase | EROD activity, i.e. catalytic formation of resorufin, in freshly isolated rainbow trout hepatocytes measured with fluorometer | I, III, V |
| *Daphnia magna* | Mobility of water fleas | I, II, III |
| *Allium cepa* | Inhibition of onion root growth | I |
| ***Fractionations*** | | |
| Modified TIE Phase I | Fractionation of effluent samples by physico-chemical methods | I |
| Methyl-*tert*-butyl ether - water extraction | Fractionation of the sample constituents into hydrophilic and hydrophobic parts | II |
| Dialysis | Assessment of the molecular weight of the toxicant using membranes with MWCO 1000, 3500 and 6000-8000 | II |

**Table 6.** Chemical and statistical methods used in the original publications relating to this study.

| Test method | Aim of the method | Publication |
|---|---|---|
| ***Chemical assessment*** | | |
| pH, suspended solids, conductivity, BOD, COD, ammonium, total N and P, hardness, AOX | Measurement of parameters often used for monitoring and controlling wastewater effluents | I |
| Gas chromatography – mass spectrometry (GC-MS) | Analysis of wood extractives | II |
| Gas chromatography – flame ionisation detector (GC-FID) | Analysis of wood extractives | II |
| UV visible spectrophotometry | Determination of lignin concentration | II, V |
| High performance liquid chromatography and photodiode array detection (HPLC-PAD) | Determination of lignin molecular weight distribution | II |
| Proton nuclear magnetic resonance ($^1$H-NMR) | Characterisation of the chemical structure of the toxicant | II |
| HPLC/ UV-Vis | Measurement of NPE and NP concentrations | V |
| HPLC-MS/electrospray ionisation (ESI) | Measurement of NPE and NP concentrations | V |
| ***Characterisation of toxic structures*** | | |
| Laboratory-scale kraft pulping | Pulping of pine chips to obtain material for bleaching | III |
| - elemental chlorine free (ECF) bleaching | Bleaching of kraft pulp (sequence O-$D_0$-EOP-$D_1$-ED) to obtain effluents with varying composition of HMW compounds | III |
| - totally chlorine free (TCF) bleaching | Bleaching of kraft pulp (sequence O-Z-Q-P-Z-Q-P-P) to obtain effluents with varying composition of HMW compounds | III |
| Pyrolysis – GC/MS | Determination of chemical structures in effluents from kraft pulping and bleaching | Kukkola *et al.*, 2006 |
| Two-tailed Pearson correlation | Correlation of results from RET test and pyrolysis | IV |
| Principal component analysis (PCA) | Multivariate analysis to explain RET results by pyrolysis results | IV |
| Stepwise multiple linear regression (SMLR) | Multivariate analysis to explain RET results by pyrolysis results | IV |

# 4 Results and discussion

## 4.1 Screening of wastewater effluents (I)

A number of municipal and industrial effluent samples were studied with basic chemical analysis and a battery of ecotoxicological tests. A spiked reference sample containing nickel, zinc, ammonium, hypochlorite and four different organic compounds was used to verify that the toxicity tests used in the study were working properly and were capable of detecting toxic effluent samples. The spiked effluent was toxic in all the toxicity tests used (Article **I**, Table 2). In addition, positive chemical controls were also routinely included in the ecotoxicity test designs in order to verify the validity of the specific test occasion.

The results with the actual effluent samples showed that the treated wastewater effluents possessed only minor acute toxic properties in bacteria (luminescence), algae (growth), daphnia (mobility) and onion tests (root growth), if any. Similar results have been reported also in earlier studies with Finnish pulping wastewaters (Ahtiainen *et al.*, 2000). Low acute toxicities of wastewaters are also essential for the well-being of microbes and efficient biological treatment at WWTPs. In contrast to toxicity, many of the studied effluents exhibited stimulating effects in microbial tests. This phenomenon could be due to increased concentration of beneficial compounds (e.g. nutrients) or due to hormesis (induction at low levels of toxicants). Similar observations with effluents have been made also in other studies (Ahtiainen *et al.*, 1996). No genotoxicity was detected in the whole effluent samples.

The results with *in vitro* tests (RET, EROD and vitellogenin) were somewhat different from the whole organism assays: In the case of the RET test especially industrial effluents were toxic, the effluent from a pulp and paper mill being the most harmful. The studied effluent samples seemed to be toxic to the fish hepatocytes at high test concentrations, but at low sample concentrations an induction in vitellogenin production was measured in most of the samples. Half of the samples affected also the cytochrome P4501A measured as EROD activity – some by activating and others by inhibiting the process. Activation and inactivation effects on fish hepatocytes caused by municipal and industrial effluents have been reported also by other authors (e.g. Gagné and Blaise, 1999; Gagné and Blaise, 2000; Grung *et al.*, 2007).

The chemical analyses on the studied effluent samples comprised of the basic parameters often used in effluent consent conditions. The results are summarised in Article **I**, Table 1. The pH values of the municipal effluents varied from 7,2 to 7,5, whereas the pH values of the industrial effluents were 6,8-8,2. Suspended solids concentration was highest in the first pharmaceutical and enzyme production effluent sample, which was taken unintended at a time of plant malfunctioning. Chemical oxygen demand and total organic carbon concentrations were clearly highest in the pulp and paper mill effluents, if the releases from the WWTP treating pharmaceutical and enzyme production wastewaters during plant malfunctioning are not counted in. The highest amount of ammonium was discharged from the medium sized WWTP. Also the two pharmaceutical and enzyme production plant effluents contained rather high amounts of ammonia and especially the total nitrogen concentration was clearly higher than in the other studied effluents.

Correlations between biological effects and certain chemical parameters have often been looked for. However, these are two fundamentally different approaches and measure different phenomena. For example in this study the two effluent samples originating from the pharma-

ceutical and enzyme production varied in both chemical and biological terms: The concentration of suspended solids and organic carbon were much higher in the first sample than in the follow-up sample and the first effluent sample was generally more harmful than the second one. On the other hand, the pulp and paper mill effluents were quite similar according to chemical parameters, although differences in biological effects were observed. Thus biological effect studies provide a means to gain additional information on the sample quality.

## 4.2 Characterisation of effluents using TIE (I, II)

The effluent samples were fractionated according to a modified TIE Phase I procedure. The reference experiment with the spiked effluent showed that the fractionation methods were working reliably (publication **I**, Figure 2a): All manipulation steps, except thiosulfate addition, reduced the toxicity of the spiked sample to some extent. The effect of hypochlorite might have been masked by the other compounds and the effluent matrix so that only minimal reduction in toxicity was observed. The role of thiosulfate in reported TIE experiments has been quite minor and its necessity in the first step of TIE could thus be questioned. The SPE columns (Oasis HLB) used in this study have proven to be suitable for preparing samples for EDA also in other studies: Grung *et al.* (2007) used Oasis HLB columns to concentrate a wide range of polar and non-polar chemicals from wastewaters and finally identified, for example, 17-β-estradiol, estriol and alkylphenols as causes of estrogenic effects and nicotine as a cause of EROD activity.

In baseline effluent samples no genotoxicity as measured with *S. typhimurium* test (Vitotox® test kit) was observed. However, the test showed slight genotoxic potential in a SPE concentrate obtained from pharmaceutical and enzyme production plant effluent at pH 3. This suggests that an acidic compound could be the cause, as Oasis HLB columns tend to retain non-ionised compounds better than ionised. Remarkable is that the sample was taken at time when the plant suffered from malfunctioning and in the subsequent control sampling no genotoxic potential could be detected. Overall the first effluent sample from pharmaceutical and enzyme production plant was more harmful than the second one when assessed based on all the bioassays used. According to these results the large bioassay battery including genotoxicity assessment that is required from the chemical industry effluents in Germany seems legitimate (BMU, 2004). Pharmaceuticals in wastewater effluents have raised increasing concern lately, as genotoxicity and chronic effects of wastewater effluents associated pharmaceuticals have been reported by several authors (e.g. Giuliani *et al.*, 1996; Legault *et al.*, 1996; Schmitt *et al.*, 2005; Quinn *et al.*, 2008). The SPE concentrated samples can be seen as a simple way to represent potential accumulation in the environment and are thus valuable tools in assessing the possible environmental effects of effluents.

In addition to the hidden genotoxic potential, the fractionation procedure gave hints on the possible toxicants especially with the effluents from the pharmaceutical and enzyme production plant and the pulp and paper mill. In case of pharmaceutical and enzyme production effluents the overall toxicity detected in the RET test could be due to metals and organic compounds, because EDTA addition and SPE (at pH 3) were effective in reducing toxicity. The observed pH dependent toxicity (Figure 4) and high ammonia concentrations of the first sampling occasion (33 mg/l) speak for additional ammonia related toxicity. At low pH values ammonia is in ionised form ($NH_4^+$) and at higher values in non-ionized and more toxic form ($NH_3$). Ammonia related toxicity has been identified using TIE from both municipal and in-

dustrial effluents (e.g. Burkhard and Jenson, 1993; Jin *et al.*, 1999). However, pH dependent toxicity could be related also to metals and organic ionisable compounds.

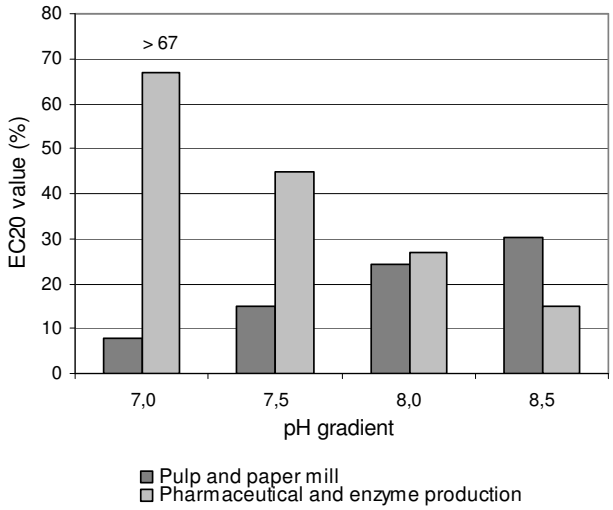



**Figure 4.** pH dependent toxicity of two effluent samples as measured with the RET assay.

In case of the pulp and paper mill effluents the superior power of the SPE in diminishing the toxic effect was clear (publication **I**, Figure 2b). Besides to SPE treatment, pH gradient and EDTA addition affected the effluent toxicity, especially when measured with the RET test. EDTA addition actually increased, not decreased the toxicity of the second effluent sample, thus eliminating metals as potential toxicants. This increase in toxicity might be due to high original EDTA concentrations that often prevail in pulp and paper mill effluents due to its use as chelator in the process. The SPE columns could, however, not retain all of the observed toxicity (publication **I**, Figure 2b). This could mean that another type of toxicant passing through the columns might be present or the columns were overloaded by the toxicant. The latter theory is supported by the fact that although the both consecutive post-column samples still exhibited toxicity, the first one was less toxic than the latter. Additional retention tests with the second pulp and paper mill effluent sample are presented in Figure 5, which clearly show the increasing overloading of the columns with larger than 2 ml samples. However, the possibility of additional toxicants still stays, as some toxicity remained even with 1 ml samples.

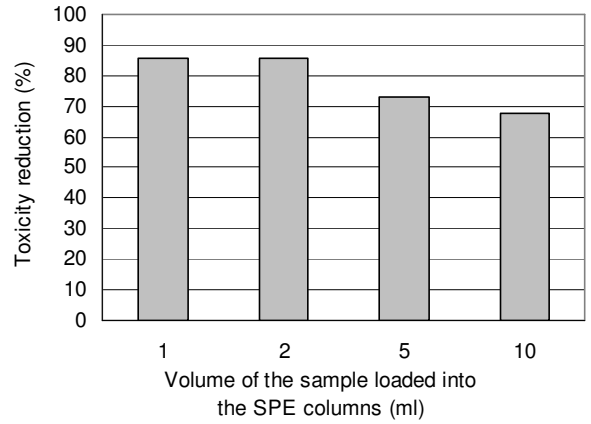



**Figure 5.** Retention tests with Oasis HLB columns and varied pulp and paper mill effluent volumes.

From the battery of tests used in the study, the RET test proved to be the most suitable to be used for EDA as the test is rapid, sensitive and reproducible. Especially the fact that the test medium is buffered favours the RET test. pH affects the ionization of compounds and thus has a direct effect on the compounds' behaviour and toxic potential. pH adjusted samples give only indicative results, when tests with no real buffering capacity are used or specific equipment is required to stabilise the conditions as is with daphnids in the original TIE procedure. For example the widely used luminescent bacteria test has this important weakness speaking against its use in the TIE procedure, as the test medium has no buffering capacity. However, the use of buffering media may also have counter effects, i.e. it may mask or alter toxic effects. pH-dependence has shown to be significant especially in cases of toxicity related to ammonia and metals (Burkhard and Jenson, 1993; Ho *et al.*, 1999; Van Sprang and Janssen, 2001). The small sample volume needed (1 ml is enough) is also in favour of RET assay. However, as an *in vitro* method it does not account for transport of compounds into cells and metabolic actions and is thus suitable only to screening and mechanistic studies.

The results of this study show that the possible impacts caused by changing pH levels have to be considered in the biological assessment of the effluents. For example in this study the pulp and paper mill effluents turned out to be more toxic at lower pH levels (Figure 4). This might be of importance if the toxicants are persistent in the environment and the pH of the receiving waters is quite low. Also the genotoxicity indicating results with the SPE concentrates speak against merely chemical, or even chemical combined with WET, assessment of effluents: With just a few very simple sample fractionations a lot of valuable additional exposure information can be gained.

Fractionation of the effluent samples using modified TIE procedure gave hints of the possible chemical characters of toxicants, revealed possible genotoxic potential in one of the effluents studied and indicated that the particular toxicant(s) in pulp and paper mill effluent may possess higher toxicity under environmentally relevant pH conditions when compared to the original discharge pH. The WWTP processing pulp and paper mill wastewater was chosen for further toxicant characterisation experiments. The RET assay was chosen as the main screening test to be used in the further steps of this study.

## 4.3 Identification of a suspect toxicant from pulp and paper mill wastewater effluent (II)

In the toxicant identification step altogether five effluent samples (two from the previous step and three additional) from the pulp and paper mill were evaluated. According to the toxicant characterisation the toxicant was likely to be an organic compound (weak acid) that responds to pH changes, being more toxic at low pH values. At this point the U.S. EPA TIE Phase II methods could be used only as indicative, as in the case of organic toxicants the methods focuses on non-polar compounds and fractionation with C18 columns (U.S. EPA, 1993a). In this study further fractionations were carried out using similar SPE columns as in the previous characterisation step.

In order to ensure the adequacy of the columns only 2 ml sample volumes were passed through one SPE column. The columns were washed with water and eluted with a methanol-water series, where methanol concentration ranged from 50 to 100 %. Toxicity was mainly recovered in the washing fraction and in the two most hydrophilic methanol-water fractions indicating that the toxicant was hydrophilic (publication **II**, Figure 1). More hydrophobic

compounds, such as wood extractives and their degradation products, have often been considered to be the most potential harmful components of pulp and paper industry wastewaters (e.g. Hewitt *et al.*, 2006). Wood extractives were used as model compounds also in this study, but they were eluted mainly with higher methanol concentrations.

The hydrophilicity and the molecular weight of the toxicant were further characterized with methyl-*tert*-butyl ether (MTBE) extraction and dialysis experiments. The MTBE experiment showed that the toxicity was mainly recovered in the water phase, giving more support of the hydrophilic nature of the toxicant. In the dialysis experiments (MWCO 1000, 3500 and 6000-8000) the toxicity could not be reduced by any of the membranes used, meaning that the toxicants would be HMW compounds.

In order to analyse the hydrophilic HMW compounds present in the pulp and paper mill effluent proton nuclear magnetic resolution ($^1$H-NMR) was used. It verified that the HMW compounds had structural characteristics of lignin. Using this information lignin concentrations in baseline effluent, as well as in SPE fractions, were determined and they revealed that lignin was present in all toxic samples.

A commercial alkali lignin preparation was used as model compound to test lignin toxicity on the RET assay. It showed RET inhibiting properties and, more important, a similar pH dependence could be found with the commercial lignin as with the effluent samples. However, definite identification and confirmation of the toxicity causing compound could not be achieved due to the nature of the potential toxicant: The commercial alkali lignin preparation served as a surrogate for testing the suspect toxicant, but is structurally different from lignin that has been processed by bleaching and wastewater secondary treatment steps.

The confirmation approaches proposed in U.S. EPA TIE Phase III were not applicable directly to this study (see chapter 1.3.2., Table 4.). The correlation approach seemed to be most suitable, although the measurement of the amount of polymer lignin is always not so straightforward. Toxicity, expressed as RET test EC50 values, was increasing with increasing lignin concentrations, when the three effluent samples originating from softwood pulping were considered. The additional two hardwood pulping effluents did not match with this correlation. However, the sample number was very limited, and thus too much value cannot be put on this.

Pulp and paper mill effluents and their biological effects have been studied for decades. During this time both the industrial processes and the biological methods have developed. However, no explicit cause for the effects still observed in the environment has been identified, but usually rather hydrophobic and small compounds such as retene, resin acids and β-sitosterol have been named as potential cause of biological effects, as reviewed by Lehtinen (2005) and Hewitt *et al.* (2006; 2008). The unveiling of hydrophilic HMW lignin as the main cause of RET inhibition in this study was quite surprising, although possible biological effects of lignin-related HMW fractions have been reported by some other researchers (Higashi *et al.*, 1992; Pillai *et al.*, 1997; Hall and LaFleur, 2003). Eklund *et al.* (1996) also showed that inhibition of the reproduction of marine red algae (*Ceramium strictum*) was caused by the hydrophilic fraction of the studied pulp mill effluents, but no further identification of the toxicant(s) was reported.

Lignin has been generally considered to be quite inert in biological terms, as it cannot be taken up by cells due to its high molecular weight. However, lignin-related small compounds

have been reported to affect, for example, the growth of fungi and activity of methanogenic bacteria as well as the reproductive performance of mummichog fish (Sierra-Alvarez and Lettinga, 1991; Buswell and Eriksson, 1994; Dekker *et al.*, 2002; Hewitt *et al.*, 2002). The RET assay, as a test system free of cell membrane is obviously especially suspect to HMW compounds, when compared to whole organisms. However, according to the results obtained in this study with commercial alkali lignin, lignin might also affect, for example, bacteria and daphnids to some extent.

Although all results pointed towards lignin as the cause of toxicity, at this point it could not be ruled out that the toxicity might be caused by other compounds attached to lignin. It has been shown that smaller size compounds can be tightly associated with lignin polymer. For example Bullock *et al.* (1996) showed that against all expectations, nitrogen (as nitrate or nitrite) may be mainly associated with the kraft pulp mill HMW fraction and only less with the low molecular weight (LMW) fraction. Also the binding of hydrophobic organic compounds to lignin has been demonstrated (Kukkonen, 1992; Kukkonen and Oikari, 1992).

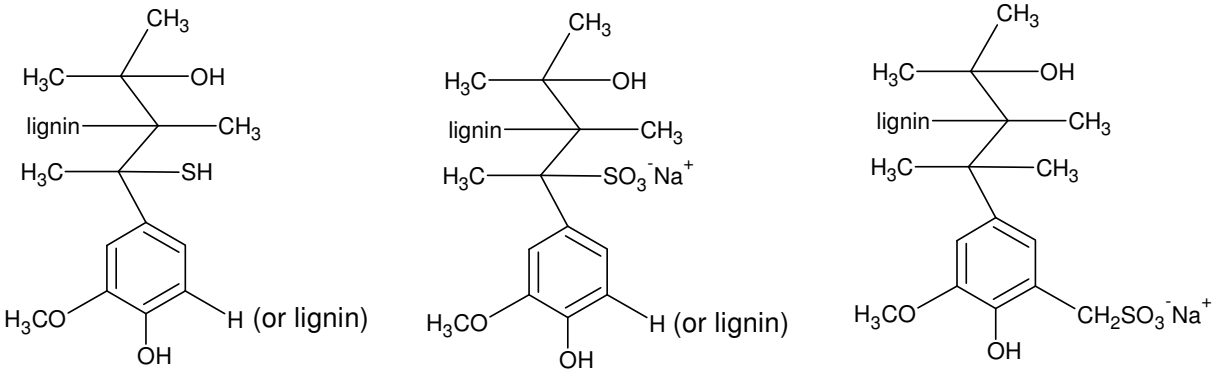
The confirmation step was further complicated by the fact that molecular weight distribution studies using HPLC revealed that the HMW compounds differed in size between the sampling occasions: The second effluent sample that had been taken just after plant start-up contained higher molecular weight compounds than the other effluents and this sample was also most toxic in the RET assay. The next step in the study was to get more information on the chemical features relating to toxicity. This might be of special relevance also for actual environmental exposure cases, especially if smaller compounds carrying structures identified to cause toxicity would be released from HMW lignin.

## 4.4 Characterisation of toxic chemical traits (III, IV)

In order to get more information on the possible structures causing inhibition in the RET assay, different lignin derivatives (commercial and in-house extracts) were evaluated for their possible biological effects. A list of the studied derivatives can be found in Article **III** (Table 1) and the chemical structures of some of the derivatives are delineated in Figure 6 of this summary. *V. fischeri*, *D. magna* and fish hepatocytes were used in addition to the RET assay. Also laboratory-scale kraft pulping with pine chips and bleaching using ECF and TCF sequences were carried out. Biological effects of the resulting effluents, and especially their HMW fractions, were studied with RET and *V. fischeri* tests.

This part of the study confirmed that HMW lignin is biologically active. Lignosulfonates were less toxic than alkali lignin in the RET assay indicating that sulfonation on the alkyl part reduces the inhibition of RET. Similarly carboxylation decreased the RET inhibiting property of alkali lignin. The impact of sulfonate groups located on the aromatic ring of alkali lignin was not distinct, as the studied derivative had only low degree of sulfonation. Similarly in the *V. fischeri* test carboxylation and sulfonation reduced the toxicity with short exposure time (30 s). After longer exposure (15 and 30 min) most of the studied derivatives did not inhibit the luminescence anymore. This may be due to nutrients enhancing the luminescence and masking the possible inhibition. *D. magna* were not affected, although in the previous step of this study they were affected by the commercial alkali lignin preparation. However, the daphnia strain was different at this point of the study and it has been demonstrated that the sensitivity of different strains may vary (Baird *et al.*, 1991; Oda *et al.*, 2007).

Most of the studied lignin derivatives were toxic to the fish hepatocytes, meaning that the EROD activity was decreasing. Reduced EROD activity and decreased vitellogenin production in the exposed hepatocytes at high test concentrations, has been considered as a cytotoxic effect and seems to be representative for the hepatocyte assay at high test concentrations (Hahn *et al.*, 1993; Anderson *et al.*, 1996; Chen and Bunce, 2003; Nakari, 2005; Nakari and Pessala, 2005). Although the derivatives were mainly toxic to the hepatocytes, the EROD results with the alkali lignin derivatives purchased from Aldrich still indicate that carboxylation reduces the toxicity of alkali lignin towards fish hepatocytes also.




Alkali lignin | Lignosulfonic acid | Sulfonated alkali lignin

**Figure 6.** Indicative chemical structures of some lignin derivatives (based on information from Sigma-Aldrich Company Ltd and Willauer *et al.*, 2000).

The toxicities of the effluents collected from the laboratory-scale pulping and bleaching experiments were studied with RET and *V. fischeri* tests. In both tests the black liquor was equally toxic, but the RET test was more sensitive towards the subsequent bleaching effluents. Toxicity of the HMW fraction, or the untreated bleaching effluents, were not dependent on the amount of lignin in the effluents. Thus, if lignin were the only toxicant present in the effluents, lignin compounds released into the effluents during different bleaching stages had to be of varying toxicity. This could be due to differences in lignin structure, as it is known from earlier studies that the dissolved lignin has different chemical characters along the bleaching sequence (Gellerstedt *et al.*, 1999). For example after oxygen stage dissolved lignin has a substantially high amount of highly substituted aromatic rings and vinylic carbons are more abundant in pulping and oxygen stage effluents when compared to peroxide stage effluents. The effluents originating from the ozone stage of TCF bleaching were especially toxic when normalized to their lignin content. However, ozone is a strong oxidizing agent that forms radicals capable to attack carbohydrates in addition to lignin (Alén, 2000). Thus, it is possible that carbohydrates had an effect on the overall toxicity of the HMW fractions.

The HMW fractions (Mw >1000) were further characterized by combined pyrolysis and GC/MS experiments (Kukkola *et al.*, 2006). Pyrolysis can be used to obtain detailed structural information on HMW matter. Due to the limitations of pyrolysis experiments to identify polar functional groups both original and methylated samples were studied. As a result from pyrolysis experiments, the relative amounts of identified compounds originating from lignin and carbohydrate matter were known for all pulping and bleaching effluents. The amounts of identified structures were compared to the corresponding toxicity values obtained from the RET assay using multivariate statistics, which has not been used earlier in this context.

Two-tailed Pearson correlation showed that the compound patterns in all samples were quite similar, except for effluents originating from the TCF ozonation step. As stated earlier, this was observed also in the toxicity pattern of the effluents. Both PCA and SMLR on TCF samples revealed that the higher toxicity of the ozone stage effluents is likely due to carbohydrate macromolecule originating structures. When methylated pyrolysates of TCF effluents were analyzed, compounds originating from lignin-related source structures of toxicity were identified, namely 2-methylphenol, 2-methylsyringol, 4-vinylsyringol, 2-methoxy-4-methylphenol, 4,5-dimethoxy-2-methylphenol. Notably all these compounds have phenolic hydroxyl structures with methyl or methoxyl groups in *ortho* position to it (Figure 7, solid spheres). However, due to the complex nature of lignin, it is quite difficult to judge which of the functional groups had been formed in the pyrolysis and which ones in the preceding methylation treatment, especially as also a partial methylation due to steric hindrances is likely to have happened (Challinor, 1995). With ECF samples the low sample number (only five bleaching steps) was limiting the use of SMLR, but with PCA styrene could be identified as the most probable lignin originating toxicity related structure. Thus, two of the identified structures had an ethylene double bond (dashed spheres).

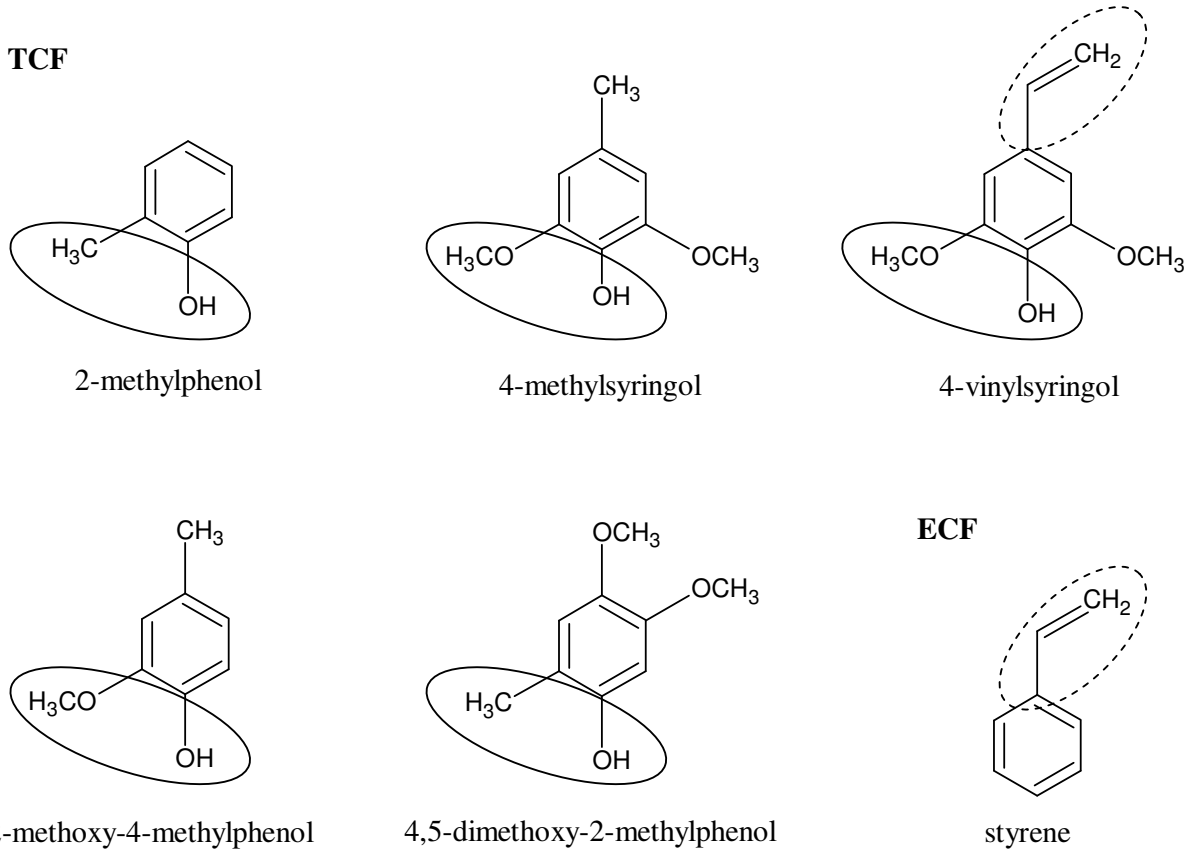



**Figure 7.** Compounds originating from source structures of lignin-related toxicity.

Earlier in this study lignin toxicity was found to be dependent on the test pH (Articles **I** and **II**). Dissolved lignin is rich in hydroxyl groups and it has been proven in several studies that the toxicity of phenolic compounds increases with the decrease in pH value and the ionization of the phenolic hydroxyl group (Svenson and Zhang, 1995; Sinclair *et al.*, 1999; Kouts *et al.*, 2005). Also the multivariate analysis indicated that phenolic hydroxyl structures with adjacent functional groups may be important in lignin related RET inhibition. This is supported by

studies indicating that substitution in *ortho* position increases the toxicity of phenolic compounds (Guiraud *et al.*, 1995; Argese *et al.*, 2005). However, also thiol groups, capable to form disulfide bonds and essential to many biological systems, may react to changing pH values. Thus, certain pH-dependent functional groups may be important for the toxic effect of lignin – a set up that may be affected, for example, by sulfonation.

## 4.5 Biodegradation of harmful organic compounds (V)

As a part of the TRE procedure carried out in the USA, TIE is conducted and possible control options are considered (U.S. EPA, 2001). If the use or processing of compounds identified as toxicants cannot be cut out, treatment alternatives to remove the toxicant from the final effluent have to be considered. In case of organic compounds one potential treatment step is microbial degradation. If the toxic effluent originates from a WWTP that already utilizes secondary treatment, one possible solution could be to extend the HRT to give more time for microbes to degrade or modify the compounds. Thus, in this step of the present study, aerobic biodegradation of two organic compounds, namely NPE and lignin that are ubiquitous in wastewater streams and may be the cause of harmful effluent properties, were studied. In addition to chemical assessment, biodegradation of the compounds was monitored with bioassays, to be able to evaluate the possibly beneficial biological effects of the biodegradation process. Similar approach could be used also for predicting possible effects of chemicals and their degradation products in the environment.

In the biodegradation studies three different inoculum sources were used. Tests with reference compound aniline showed that the source of inoculum plays an important role in the efficiency and rate of degradation: Inocula from municipal WWTPs were obviously adapted to aniline type of compounds, as the mineralization of aniline started immediately with no lag time (publication **V**, Figure 4). With an inoculum from a pulp and paper mill, on the other hand, the mineralization started only after a lag time of one week. The capability of microbes to mineralize harmful compounds in short time is extremely important for the proper functioning of WWTPs. Thus, harmful compounds appearing only occasionally at the biological unit are tricky, as the biomass may not adapt to these compounds. Reduction of the use of harmful compounds is certainly recommended, but may periodically result also in reduced effluent quality due to the lack of adapted microbes.

The biodegradation of NPEs was studied using the municipal WWTP inocula. Mineralization of the commercial NPE mixture varied between the two experiments, the inoculum from Helsinki City WWTP being more efficient. In these experiments NPE mineralization was clearly lower when compared to a study conducted by U.S. researchers (Staples *et al.*, 2001), where approximately 70 % mineralization was observed. It could be speculated that NPE concentrations in Finnish wastewaters are lower when compared to USA, which results in non-adapted sludge microbes being less prone to degrade NPEs. In this study the more detailed chemical analyses on primary degradation using HPLC-MS/ESI revealed a shift in the ethoxylate chain length from longer to shorter soon after the beginning of the NPE biodegradation tests, the total NPE concentration being dropped to a tenth of the original after 3 days of incubation. In addition, in the biodegradation test with the Helsinki City WWTP inoculum also NP was formed, which is usually expected to be formed only under anaerobic conditions. However, a lot of unidentified compounds other than short chain NPEs, NP and $CO_2$ were formed and according to earlier studies, they are likely to be carboxylated APEs (Staples *et al.*, 2001; Langford *et al.*, 2005).

At the same time with the decrease in total NPE concentration also toxicity (RET) and estrogenic activity (hER yeast) measured directly from the biodegradation liquors decreased. With the fish hepatocytes no clear effect of the studied NPE mixture could be measured due to the possible interference of the inoculum and/or test medium. The inoculum used in this particular case originated from a large municipal WWTP that had showed vitellogenin inducing properties already in the beginning of this study (Article **I**).

The more hydrophobic short chain metabolites of the NPE degradation have generally been considered to be more harmful than the parent compounds and especially the estrogenic potential of NP has been the main concern (Ahel *et al.*, 1994; White *et al.*, 1994; Routledge and Sumpter, 1996). This was not the case in this study as the biological effects diminished along the degradation and shortening of the chain length. Still it is possible that the concentration of the potentially (more) toxic degradation products was too low to cause observable effects. It is also possible that hydrophobic metabolites are attached to particles being not bioavailable. However, an earlier study on commercially available NPE mixture, NP and nonylphenoxy carboxylic acid (Argese *et al.*, 1994) support findings of this study, i.e. the parent NPEs being somewhat more harmful than the degradation products. Argese *et al.* (1994) reported the following EC50 values for RET assay: NPE mixture 1,3 mg/l, NP 1,8 mg/l and nonylphenoxy carboxylic acid 8,2 mg/l. The slight estrogenic potential of the NPE mixture in hER yeast has also been detected by other authors (Andersen *et al.*, 1999; Isidori *et al.*, 2006).

In the lignin degradation experiment only approximately 11 % mineralization was achieved. However, according to the chemical analyses lignin concentration had decreased by 8-30 % percentages during the 4-week incubation, indicating that biotransformation was indeed happening. This is supported by the RET assay results, as the toxicity started to decrease in the beginning of the study, but later seemed to be increasing again. Early works using $^{14}C$-labelled lignocellulose have shown that actinomycetes may produce small-size molecules and HMW matter with markedly elevated number of phenolic hydroxyl and carboxylic acid groups as reviewed by Vicuña (1988). Also the mineralization of lignin by bacteria was referred to be near 10 %. This is consistent with results obtained in this study. Recently it has been reported that aerobic bacterial treatment of commercially available kraft lignin (Sigma-Aldrich) has resulted especially in the formation of some acidic low molecular weight compounds (Raj *et al.*, 2007). Thus the tendency of toxicity to increase in this biodegradation study could be due to the formation or release of harmful LMW compounds and/or increasing number of toxicity related structures in HMW lignin. According to these results it seems that longer secondary treatment of lignin alone would not be a recommended control option. Enhanced removal of lignin or its degradation products, for example by adsorption onto sludge, would be needed. However, this study was carried out with commercially available kraft lignin, which differs to certain extent from dissolved lignin in bleaching effluents that are directed to the WWTPs.

Studies linking bioassays directly (or with only filtration treatment) to any standardized aquatic biodegradation test are quite scarce. The low level of toxicants sets obvious limitations to this approach, as the toxicant concentrations may not be toxic to the degrading bacteria but have to have an effect in the bioassay. Recently, Stasinakis *et al.* (2008) studied compounds possessing endocrine disrupting potential by combining a biodegradation test (manometric respirometry test, OECD 301F) to acute toxicity test (*V. ficheri*). In this study the toxicity of, for example, NP decreased during the 4-week incubation, whereas the toxicity of studied perfluoro compounds increased. The study reported by Kümmerer *et al.* (2000) is an-

other example of the successful combination of the biodegradation test and a small-scale biotest. They studied the genotoxicity of several pharmaceuticals. Based on some earlier reports and this present study, it seems that direct biological assessment of biodegradation intermediates from the biodegradation liquors is possible, if sensitive bioassays are used. In addition, the test medium and inoculum itself should not cause too much background effects in the tests. Thus, of the tests used in this study, RET and hER yeast assays proved to be most suitable. However, if a tiered approach to reveal the exact toxicity causing compounds is planned, a more concentrate sample would be beneficial. A similar approach that has been used earlier in this study to characterize the toxicants and toxicity related structures could also be used to assess the changes in toxicants during biodegradation processes.

# 5 Summary and conclusions

Screening of wastewater effluents from municipal and industrial WWTPs with biotests showed that the treated wastewater effluents possess only minor acute toxic properties towards whole organisms (e.g. bacteria, algae, daphnia), if any. *In vitro* tests (RET and fish hepatocytes) were generally more susceptible to the effluents. Most of the effluents indicated the presence of hormonally active compounds, as the production of vitellogenin, an egg yolk precursor protein, was induced in fish hepatocytes. In addition, indications of slight genotoxic potential was found in one effluent concentrate with a recombinant bacteria test.

The biological effects observed could not have been predicted using only routine chemical effluent monitoring parameters. Therefore chemical parameters cannot be considered to be sufficient in controlling effluent discharges especially in case of unknown, possibly bioaccumulative, compounds that may be present in small concentrations and may cause chronic effects. In addition, mixture toxicity may be somewhat different than information gained for single substances, which, however, is usually used for risk assessments. Thus in the case of wastewaters, i.e. extremely complex aquatic mixtures, the biological assessment seems to be essential.

Throughout this study the RET test was used as a model test to conduct effluent assessment followed by toxicant characterisations and identifications. Using a modified U.S. EPA TIE Phase I scheme and additional case-specific methods, the main compound in a pulp and paper mill effluent causing RET inhibition was characterised to be an organic, relatively hydrophilic HMW compound. The toxicant could be verified as HMW lignin by structural analyses using nuclear magnetic resonance. In the confirmation step commercial and in-house extracted lignin products were used. The possible toxicity related structures were characterised by statistical analysis of the chemical breakdown structures of laboratory-scale pulping and bleaching effluents and the toxicities of these effluents. Finally, the biological degradation of the identified toxicant and other wastewater constituents was evaluated using bioassays in combination with chemical analyses. The compounds resulting from a biodegradation process can be characterised and identified using the same strategy and procedure as with the effluents.

Bioassays to be used for screening effluent quality should cover a range of endpoints, and be sensitive, cost-effective and rapid. From the battery of tests used in the study, the RET assay proved to be most suitable for effluent quality monitoring. Other potential methods could be recombinant microbes measuring, for example, genotoxic and hormonal effects. In the future also microarray techniques can provide convenient assessment tools. Sensitive bioassays should be used as tools to control and monitor the effluent properties. Biological effects of the potentially bioaccumulative components should also be estimated using, for example, rapid concentration methods such as SPE. If biological responses were found, the harmful compound(s) should be characterised, identified and quantified, if possible. After this compound-specific risk assessment should be conducted, using, for example, whole organisms and experiments simulating environmental conditions. If environmental consequences are likely, consideration of possible control options to diminish or cut out the discharges should be carried out. Monitoring of the effluent quality by biological methods should be carried out to verify the efficiency of the managerial decisions.

The tiered approach proposed in this study that combines chemical, biological and statistical methods is summarised in Figure 8. It provides tools for evaluating wastewater effluents with ecotoxicological methods, in addition to characterising and identifying their toxicity causing compounds and structures.

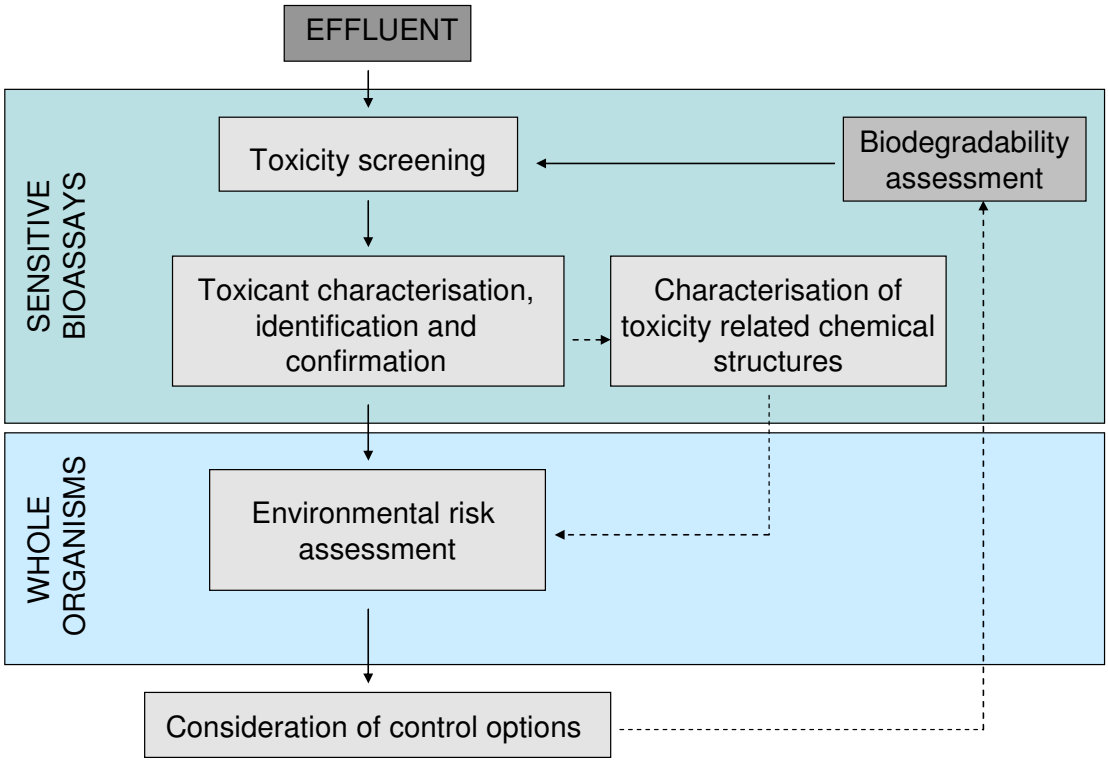



**Figure 8.** A scheme for evaluating biologically active organic compounds appearing in wastewater effluents.

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